

DECLARATION

I, Yasuhiko Kinomura, residing at 10-4, Higashitomigaoka 5-chome, Nara-shi, NARA 631-0002 JAPAN, and a citizen of Japan, declare the following:

(1) I am fluent in both the English and Japanese languages and capable of translating documents from English into Japanese and from Japanese into English.

(2) I am a translator at Iwatani Patent Office, whose address is Orix Dojima Build. 3F, 1-31, Dojima, 2-Chome, Kita-ku, Osaka, 530-0003, Japan.

(3) U.S. Patent Application No. 10/500,018 is the national stage application of International Patent Application No. PCT/JP03/04590, filed April 10, 2003. International Patent Application No. PCT/JP03/04590 was filed in the Japanese language and corresponds to WO 03/084983.

(4) In the translation of PCT/JP03/04590 from Japanese to English, the Japanese characters “又は” were inadvertently and mistakenly translated into the word “and” in U.S. Patent Application 10/500,0018 as filed. It is the object of this declaration to correct the translation error with respect to the Japanese characters “又は” which mean “or” not “and.”

(6) The translation error as described herein appears in the specification and claims of U.S. Patent Application No. 10/500,018 and corresponds to the specification pages and claims of PCT/JP03/04590 as shown in the following table:

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U.S. Patent Application No. 10/500,018	PCT/JP03/04590 (WO 03/084983)
In the specification at page 23, line 13	In the specification at page 18, line 1
In the specification at page 24, line 27	In the specification at page 19, line 5
Original Claim 13 (page 107, line 11)	Original Claim 13 (page 87, line 16)
Original Claim 19 (page 109, line 2)	Original Claim 19 (page 88, line 26)
Pending Claim 35	
Pending Claim 41	

(7) To the best of my knowledge, **Appendix A** attached herein is a true and accurate English translation of pages 23-24 and claims 13, 19, 35, and 41 of U.S. Patent Application No. 10/500,018.

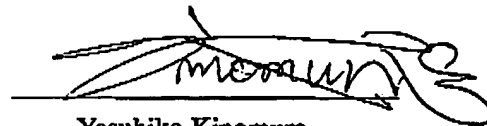
(8) To the best of my knowledge, **Appendix B** attached herein is a true and accurate copy of pages 18-19 and claims 13 and 19 of PCT/JP03/04590 (WO 03/084983).

(9) This amendment does not introduce new matter.

(10) I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

I declare under penalty of perjury that the foregoing is true and correct.

Signature:



Yasuhiko Kinomura

Dated:

June 16, 2006

# APPENDIX A

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according to any one of the above (9) to (12), which comprises producing a protected peptide fragment containing no modified amino acid or non-amino acid, by an enzymatic method or/and a genetic recombination method,

(14) the method for producing a modified peptide or protein according to the above (13), wherein the protected peptide fragment containing no modified amino acid or non-amino acid is produced by a method comprising;

step (1); a step of culturing a cell transformed with an expression vector having one of a nucleotide sequence encoding a peptide having an amino acid sequence of the peptide fragment (hereinafter, referred to as desired peptide in the present item) or a nucleotide sequence encoding a fusion protein optionally with a protective peptide added to the desired peptide via a linker sequence, and collecting the desired peptide or the fusion protein from the culture;

step (2); a step of cleaving and separating the protective peptide and, optionally, a linker sequence and the desired peptide from the resulting fusion protein, and optionally further purifying the desired peptide when the fusion protein is collected in the step (1); and

step (3); a step of protecting, with a protecting group, one or more reactive functional groups which may cause an undesirable side reaction, selected from the group consisting of a hydroxy group, an amino group, a guanidino group, imidazolyl group, an indolyl group, a mercapto group and a carboxyl group, in the side chain of the desired peptide obtained in the step (1) or the step (2),

(15) the method for producing a modified peptide or protein according to the above (14), wherein cleavage and separation of the protective peptide and, optionally, the linker sequence and the desired peptide in the step (2) is performed at two steps using an OmpT protease or a derivative thereof and a Kex2 protease or

a derivative thereof,

(16) the method for producing a modified peptide or protein according to the above (14) or (15), wherein the linker sequence is a sequence set forth in SEQ ID NO: 27,

(17) the method for producing a modified peptide or protein according to any one of the above (13) to (16), wherein the peptide fragment is a peptide fragment containing no modified amino acid or non-amino acid in ghrelin or a derivative thereof,

(18) the method for producing a modified peptide or protein according to any one of the above (13) to (17), wherein the protected peptide fragment containing no amino acid or non-amino acid is purified and stored in a solution having a pH of 4 to 8,

(19) the method for producing a modified peptide or protein according to any one of the above (13) to (18), wherein the protecting group is a Boc group,

(20) a method for producing a protected peptide fragment containing no modified amino acid or non-amino acid, which comprises producing the peptide fragment by a method comprising:

step (1); a step of culturing a cell transformed with an expression vector having one of a nucleotide sequence encoding a peptide having the desired amino acid sequence (hereinafter, referred to as desired peptide in the present item) or a nucleotide sequence encoding a fusion protein optionally with a protective peptide added to the desired peptide via a linker sequence, and

recombination method.

13. The method for producing a modified peptide or protein according to claim 12, wherein the protected peptide fragment containing no modified amino acid or non-amino acid is produced by a method comprising;

step (1); a step of culturing a cell transformed with an expression vector having one of a nucleotide sequence encoding a peptide having an amino acid sequence of the peptide fragment (hereinafter referred to as desired peptide, in the present claim 13) or a nucleotide sequence encoding a fusion protein optionally with a protective peptide added to the desired peptide via a linker sequence, and collecting the desired peptide or the fusion protein from the culture;

step (2); a step of cleaving and separating the protected peptide and, optionally, the linker sequence and the desired peptide from the resulting fusion protein, and optionally further purifying the desired peptide when the fusion protein is collected in the step (1); and

step (3); a step of protecting, with a protecting group, one or more reactive functional groups which may cause an undesirable side reaction, selected from the group consisting of a hydroxy group, an amino group, a guanidino group, an imidazolyl group, an indolyl group, a mercapto group and a carboxyl group, in the side chain of the desired peptide obtained in the step (1) or the step (2).

14. The method for producing a modified peptide or protein according to claim 13, wherein cleavage and separation of the

protective peptide and, optionally, the linker sequence and the desired peptide in the step (2) is performed at two steps using an OmpT protease or a derivative thereof and Kex2 protease or a derivative thereof.

15. The method for producing a modified peptide or protein according to claim 13 or 14, wherein the linker sequence is a sequence set forth in SEQ ID NO: 27.

16. The method for producing a modified peptide or protein according to any one of claims 12 to 15, wherein the peptide fragment is a peptide fragment containing no modified amino acid or non-amino acid in ghrelin or a derivative thereof.

17. The method for producing a modified peptide or protein according to any one of claims 12 to 16, wherein the protected peptide fragment containing no modified amino acid or non-amino acid is purified and stored in a solution having a pH of 4 to 8.

18. The method for producing a modified peptide or protein according to any one of claims 12 to 17, wherein the protecting group is a Boc group.

19. A method for producing a protected peptide fragment containing no modified amino acid or a non-amino acid, which comprises producing the peptide fragment by a method comprising:  
step (1); a step of culturing a cell transformed with an expression vector having one of a nucleotide sequence encoding

a peptide having the desired amino acid sequence (hereinafter, referred to as desired peptide, in the present claim 19) or a nucleotide sequence encoding a fusion protein optionally with a protective peptide added to the desired peptide via a linker sequence, and collecting the desired peptide or the fusion protein from the culture;

step (2); a step of cleaving and separating the protective peptide and, optionally, the linker sequence and the desired peptide from the resulting fusion protein and, optionally further purifying this, when the fusion protein is collected in the step (1);

step (3); a step of protecting, with a protecting group, one or more reactive substituents which may cause an undesirable side reaction, selected from the group consisting of a hydroxy group, an amino group, a guanidino group, an imidazolyl group, an indolyl group, a mercapto group and a carboxyl group, in the side chain of the desired peptide obtained in the step (1) or (2); and

step (4); a step of purifying and storing the protected desired peptide obtained in the step (3) in a solution having a pH of 4 to 8.

20. The method for producing a protected peptide fragment containing no modified amino acid or non-amino acid according to claim 19, wherein the protecting group is a Boc group.

21. The method for producing a protected peptide fragment containing no modified amino acid or non-amino acid according to claim 19 or 20, wherein cleavage and separation of the



Claim 35. The method for producing a modified peptide or protein according to claim 34, wherein the protected peptide fragment containing no modified amino acid or non-amino acid is produced by a method comprising;

step (1); a step of culturing a cell transformed with an expression vector having one of a nucleotide sequence encoding a peptide having an amino acid sequence of the peptide fragment (hereinafter referred to as desired peptide, in the present claim 35) or a nucleotide sequence encoding a fusion protein optionally with a protective peptide added to the desired peptide via a linker sequence, and collecting the desired peptide or the fusion protein from the culture;

step (2); a step of cleaving and separating the protected peptide and, optionally, the linker sequence and the desired peptide from the resulting fusion protein, and optionally further purifying the desired peptide when the fusion protein is collected in the step (1); and

step (3); a step of protecting, with a protecting group, one or more reactive functional groups which may cause an undesirable side reaction, selected from the group consisting of a hydroxy group, an amino group, a guanidino group, an imidazolyl group, an indolyl group, a mercapto group and a carboxyl group, in the side chain of the desired peptide obtained in the step (1) or the step (2).

Claim 41. A method for producing a protected peptide fragment containing no modified amino acid or non-amino acid, which comprises producing the peptide fragment by a method comprising:

step (1); a step of culturing a cell transformed with an expression vector having one of a nucleotide sequence encoding a peptide having the desired amino acid sequence (hereinafter,

referred to as desired peptide, in the present claim 41) or a nucleotide sequence encoding a fusion protein optionally with a protective peptide added to the desired peptide via a linker sequence, and collecting the desired peptide or the fusion protein from the culture;

step (2); a step of cleaving and separating the protective peptide and, optionally, the linker sequence and the desired peptide from the resulting fusion protein and, optionally further purifying this, when the fusion protein is collected in the step (1);

step (3); a step of protecting, with a protecting group, one or more reactive substituents which may cause an undesirable side reaction, selected from the group consisting of a hydroxy group, an amino group, a guanidino group, an imidazolyl group, an indolyl group, a mercapto group and a carboxyl group, in the side chain of the desired peptide obtained in the step (1) or (2); and

step (4); a step of purifying and storing the protected desired peptide obtained in the step (3) in a solution having a pH of 4 to 8.

## **APPENDIX B**

において目的ペプチドという。)をコードする塩基配列又は目的ペプチドに所望によりリンカー配列を介して保護ペプチドが付加されている融合蛋白質をコードする塩基配列のいずれかを有する発現ベクターにより形質転換された細胞を培養して、当該培養物から目的ペプチド又は前記融合蛋白質を採取す

5    る工程；

工程（２）；工程（１）において融合蛋白質を採取した場合、得られた融合蛋白質から、保護ペプチド及び所望によりリンカー配列と目的ペプチドとを切断分離し、所望により目的ペプチドをさらに精製する工程；

10    工程（３）；工程（１）又は（２）で得られた目的ペプチドの側鎖における、水酸基、アミノ基、グアニジノ基、イミダゾリル基、インドリル基、メルカプト基及びカルボキシル基からなる群から選ばれる１種以上の、望ましくない副反応を惹起する可能性を有する反応性置換基を保護基により保護する工程；

15    を含む方法により製造することからなる前記（１３）に記載の修飾ペプチド又は蛋白質の製造方法、

（１５） 工程（２）における保護ペプチド及び所望によりリンカー配列と目的ペプチドとの切断分離が、Omp Tプロテアーゼ又はその誘導体及びKex 2プロテアーゼ又はその誘導体を用いて２段階で行われることからなる前記（１４）に記載の修飾ペプチド又は蛋白質の製造方法、

20    （１６） リンカー配列が、配列番号２７に記載の配列である前記（１４）又は（１５）に記載の修飾ペプチド又は蛋白質の製造方法、

（１７） ペプチド断片が、グレリンもしくはその誘導体の中の修飾をうけたアミノ酸又は非アミノ基を含まないペプチド断片であることを特徴とする前記（１３）～（１６）に記載の修飾ペプチド又は蛋白質の製造方法、

25    （１８） 修飾をうけたアミノ酸又は非アミノ酸を含まない保護されているペプチド断片を、pH 4～8の溶液中で精製及び保存することを特徴とす

る前記（１３）～（１７）に記載の修飾ペプチド又は蛋白質の製造方法、

（１９） 保護基がBoc基である前記（１３）～（１８）に記載の修飾ペプチド又は蛋白質の製造方法、

（２０） 工程（１）；所望のアミノ酸配列を有するペプチド（以下、本項  
5 において目的ペプチドという。）をコードする塩基配列又は目的ペプチドに所  
望によりリンカー配列を介して保護ペプチドが付加されている融合蛋白質を  
コードする塩基配列のいずれかを有する発現ベクターにより形質転換された  
細胞を培養して、当該培養物から目的ペプチド又は前記融合蛋白質を採取す  
る工程；

10 工程（２）；工程（１）において融合蛋白質を採取した場合、得られた融合蛋  
白質から、保護ペプチド及び所望によりリンカー配列と目的ペプチドとを切  
断分離し、所望によりさらに精製する工程；

工程（３）；工程（１）又は（２）で得られた目的ペプチドの側鎖における、  
水酸基、アミノ基、グアニジノ基、イミダゾリル基、インドリル基、メルカ  
15 プト基及びカルボキシル基からなる群から選ばれる１種以上の、望ましくな  
い副反応を惹起する可能性を有する反応性置換基を保護基により保護する工  
程；

工程（４）；工程（３）で得られた保護されている目的ペプチドを、pH 4～  
8の溶液中で精製及び保存する工程；

20 を含む方法により製造することを特徴とする修飾をうけたアミノ酸又は非ア  
ミノ酸を含まない保護されているペプチド断片を製造する方法、

（２１） 保護基がBoc基である前記（２０）に記載の修飾をうけたア  
ミノ酸又は非アミノ酸を含まない保護されているペプチド断片を製造する方  
法、

25 （２２） 工程（２）における保護ペプチド及び所望によりリンカー配列  
と目的ペプチドとの切断分離が、Omp Tプロテアーゼ又はその誘導体及び

11. 縮合剤が、ジイソプロピルカルボジイミド (DIPC)、ジシクロヘキシルカルボジイミド (DCC) 又は 1-エチル-3-(3-ジメチルアミノプロピル) カルボジイミド (EDC) であり、前記縮合剤を用いるペプチド断片 (a) と (b) の縮合が、1-ヒドロキシベンゾトリアゾール (HOBt)、1-ヒドロキシスクシンイミド (HOSu) 又は 3, 4-ジヒドロ-3-ヒドロキシー-4-オキソ-ベンゾトリアジン (HOOBt) の存在下で行われることからなる請求の範囲第9項に記載の修飾ペプチド又は蛋白質の製造方法。

12. 修飾をうけたアミノ酸又は非アミノ酸を含まない保護されているペプチド断片を、酵素法又は/及び遺伝子組換え法により製造することからなる請求の範囲第8項～第11項に記載の修飾ペプチド又は蛋白質の製造方法。

13. 修飾をうけたアミノ酸又は非アミノ酸を含まない保護されているペプチド断片を、下記方法；

15 工程 (1)；前記ペプチド断片のアミノ酸配列を有するペプチド (以下、本項において目的ペプチドという。) をコードする塩基配列、又は目的ペプチドに所望によりリンカー配列を介して保護ペプチドが付加されている融合蛋白質をコードする塩基配列のいずれかを有する発現ベクターにより形質転換された細胞を培養して、当該培養物から目的ペプチド又は前記融合蛋白質を採取

20 する工程；

工程 (2)；工程 (1) において融合蛋白質を採取した場合、得られた融合蛋白質から、保護ペプチド及び所望によりリンカー配列と目的ペプチドとを切断分離し、所望により目的ペプチドをさらに精製する工程；

工程 (3)；工程 (1) 又は (2) で得られた目的ペプチドの側鎖における、

25 水酸基、アミノ基、グアニジノ基、イミダゾリル基、インドリル基、メルカプト基及びカルボキシル基からなる群から選ばれる1種以上の、望ましくな

い副反応を惹起する可能性を有する反応性官能基を保護基により保護する工程；

を含む方法により製造することからなる請求の範囲第12項に記載の修飾ペプチド又は蛋白質の製造方法。

5

14. 工程(2)における保護ペプチド及び所望によりリンカー配列と目的ペプチドとの切断分離が、OmpTプロテアーゼ又はその誘導体及びKex2プロテアーゼ又はその誘導体を用いて2段階で行われることからなる請求の範囲第13項に記載の修飾ペプチド又は蛋白質の製造方法。

10

15. リンカー配列が、配列番号27に記載の配列である請求の範囲第13項又は第14項に記載の修飾ペプチド又は蛋白質の製造方法。

16. ペプチド断片が、グレリンもしくはその誘導体の中の修飾をうけたアミノ酸又は非アミノ基を含まないペプチド断片であることを特徴とする請求の範囲第12項～第15項に記載の修飾ペプチド又は蛋白質の製造方法。

17. 修飾をうけたアミノ酸又は非アミノ酸を含まない保護されているペプチド断片を、pH4～8の溶液中で精製及び保存することを特徴とする請求の範囲第12項～第16項に記載の修飾ペプチド又は蛋白質の製造方法。

18. 保護基がBoc基である請求の範囲第12項～第17項に記載の修飾ペプチド又は蛋白質の製造方法。

19. 工程(1)；所望のアミノ酸配列を有するペプチド（以下、本項において目的ペプチドという。）をコードする塩基配列又は目的ペプチドに所望

# EXHIBIT 1



(19)



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(11)

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(12)

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(54) **NOVEL PEPTIDES**

(57) The present invention provides a novel peptide-type compound which induces secretion of growth hormone and which has the activity of increasing the intracellular calcium ion concentration, wherein at least one

amino acid is replaced by a modified amino acid and/or a non-amino acid compound, or a pharmaceutically acceptable salt thereof.

**EP 1 197 496 A1**

## Description

## Technical Field

5 [0001] The present invention relates to a novel peptide having the action of increasing the intracellular calcium concentration or the activity of inducing secretion of growth hormone, wherein an amino acid in the peptide is modified. Further, the present invention relates to a method for obtaining said novel peptide and a method for producing the same, a gene coding said peptide or a precursor of said peptide, and a method for producing said peptide or a precursor of said peptide by use of said gene. Further, the present invention relates to a structural analogue of the novel modified peptide disclosed in the present invention, which binds to a receptor for a growth hormone secretion-inducing compound thereby exhibiting the action of increasing the intracellular calcium concentration or the activity of inducing secretion of growth hormone, as well as a method for producing the same. Further, the present invention relates to a pharmaceutical composition or a growth promoter for animals comprising said peptide or said peptide analogue as an active ingredient, as well as an antibody to said peptide or a method of utilizing the same.

## Background Art

[0002] Growth hormone (abbreviated hereinafter to GH) is a proteinous hormone synthesized in adenohypophysis and indirectly promotes growth of bone and differentiation of adipocytes and chondrocytes, and its secretion is promoted by growth hormone-releasing hormone (GHRH) and inhibited by somatostatin [J. Kendrew, et al., Eds., The Encyclopedia of Molecular Biology (Blackwell Science Ltd., London, 1994), p. 462]. GH has not only a growth-promoting action but also actions such as promotion of protein synthesis in various tissues, stimulation of transfer of depot fats and elevation of glycogen content in muscles, and a reduction in GH secretion induces dwarfism, while excessive secretion thereof induces gigantism or acromegaly [Iwanami's Dictionary of Biology, fourth edition, edited by Ryuichi Yasugi, et al. (Iwanami Syoten, Tokyo, 1997), p. 757].

[0003] Since human GH has been produced by genetic engineering, GH is used not only for treatment of dwarfism [J. O. Jorgensen, Endocr. Rev. 12, 189 (1991)], but also for treatment of other diseases, and its various effects were found [J. O. Jorgensen, et al., Horm. Res. 42, 235 (1994)]. For example, such effects include activation of reconstitution of osteoblasts and bone in the normal [K. Brixen, et al., Miner. Res. 5, 609 (1990)], enhancement of muscular strength and muscular amount in GH-deficient adults [R. C. Cuneo, et al., J. Appl. Physiol. 70, 688 (1991)], improvement of motility in GH-deficient adults [R. C. Cuneo, et al., J. Appl. Physiol. 70, 695 (1991)], remedy of heavy burns in children [D. N. Herndon, et al., Ann. Surg. 212, 424 (1990)], its combined use with gonadotropins in induction of ovulation [R. Homburg, et al., Clin. Endocrinol. (Oxf). 32, 781 (1990)], prevention of metabolic disorder by administration of prednisone [F. F. Horber and M. W. Haymond, J. Clin. Invest. 86, 265 (1990)], promotion of T cell "education" in heavy immune disorder [W. J. Murphy, et al., Proc. Natl. Acad. Sci. U. S. A. 89, 4481 (1992)], and the effect of inhibiting reduction of the body weight of the aged and the effect of enlarging adipose fat tissues and preventing dermal atrophy [D. Rudman, et al., N. Engl. J. Med. 323, 1 (1990)].

[0004] Administration of recombinant GH is effective for promotion of growth in children and normalization of defects in metabolism and functions accompanying GH-deficiency in adults, but there are problems that GH has dose-restricting side effects, cannot be orally administered and is expensive [B. A. Lefker, et al., in Growth Hormone Secretagogues in Clinical Practice, B. B. Bercu and R. F. Walker, Eds. (Marcel Dekker, Inc., New York, 1998), pp. 107-108]. Many adult patients suffer from side effects such as arthralgia and a carpal tunnel syndrome considered to be attributable to pool of excess sodium and humor, so that GH administration cannot be continued [E. Corpas, et al., Endocr. Rev. 14, 20 (1993)]. These side effects are correlated with a non-physiological pattern of hormone secretion by GH administration, and in GH administration, the pulsatility of normal GH secretion cannot be imitated [B. A. Lefker, et al., in Growth Hormone Secretagogues in Clinical Practice, B. B. Bercu and R. F. Walker, Eds. (Marcel Dekker, Inc., New York, 1998), pp. 107-108].

[0005] The pulsatility of *in vivo* GH secretion is established basically by interaction between two regulating factors derived from hypothalamus; that is, GHRH and somatostatin act on pituitary gland to regulate GH secretion [G. S. Tannenbaum and N. Ling, Endocrinology 115, 1952 (1984), R. G. Clark and I. C. Robinson, Endocrinology 122, 2675 (1988)]. The normal pattern of GH secretion differs during the day and night, and during the night, a larger amount of GH is released more frequently. The amplitude of GH release pulse is further regulated by feedback by various steroid hormones, neurotransmitters, GH and insulin-like growth factor, by nutritional status, sleep and motility [J. S. Strobl and M. J. Thomas, Pharmacol. Rev. 46, 1 (1994)].

55 [0006] To overcome the side effects caused by GH administration, a large number of compounds having a GH secretion-inducing action were synthesized, and as growth hormone secretagogue (GHS), their structural activity correlation, their pharmacology and clinical applications were extensively studied. First, peptides such as GHRP-6 (Growth Hormone-Releasing hexapeptide) were synthesized and developed as therapeutic agents for treating disorders attrib-

utable to deficiency or reduction in GH [C. Y. Bowers, et al., *Endocrinology* 114, 1537-1545(1984)]. However, because these peptide compounds could demonstrate their effect through intravenous injection only, non-peptide compounds having low-molecular weight capable of oral administration were developed [R. G. Smith, et al., *Science* 260, 1640-1643 (1993)], and some of them have advanced to a phase I clinical test [A. A. Patchett, et al., *Proc. Natl. Acad. Sci. U.S.A.* 92, 7001-7005 (1995)].

**[0007]** A series of information transfer from signal reception of receptor to functional expression is called signal transduction, and the signal transduction system coupled with G protein proceeds in the following mechanism [Iwanami's Dictionary of Biology, fourth edition, ed. by Ryuichi Yasugi, et al., pp. 555-556 (Iwanami Syoten, Tokyo, 1997)]. This G protein coupled system has a receptor with seven transmembrane domains and is divided into a cAMP system for producing cAMP as a second messenger and inositol-1,4,5-triphosphoric acid (IP3) and diacyl glycerol (DG) inositol phospholipid information transduction system. The cAMP activates cAMP-dependent kinase (A kinase), to cause phosphorylation of serine and threonine residues in functional protein to modify its activity. On the other hand, IP3 binds to IP3 receptor on endoplasmic reticulum to promote release of calcium ions, while DG activates C kinase to promote the action of hormones etc.

**[0008]** The mechanism of increasing the intracellular calcium ion concentration in the signal transduction system with IP3 or DG as second messenger [J. Kendrew, et al., Eds., *The Encyclopedia of Molecular Biology* (Blackwell Science Ltd., London, 1994), p.136-137] is as follows: When a ligand binds to the receptor, phospholipase C is activated via G protein, to convert PIP2 into IP3. By IP3, calcium ions pooled in endoplasmic reticulum (ER) as intracellular granule are released into cytoplasm, thus increasing calcium ion levels in the cytoplasm. If IP3 or calcium ions are present in the cytoplasm, the calcium is incorporated again into the endoplasmic reticulum, thus lowering calcium ion levels in the cytoplasm. That is, the binding of the ligand to the receptor causes a transient increase in calcium ion levels in the cytoplasm.

**[0009]** Since GHS acts synergistically on the GH secretion and increase of intracellular cAMP levels by GHRH [K. Cheng, et al., *Endocrinology* 124, 2791-2798 (1989)] and the binding of GHRH to the receptor induces production of cAMP as second messenger while GHS induces an increase in the intracellular calcium ion concentration, it was suggested that the working mechanism of GHS is different from that of GHRH [J. Herrington and B. Hille, *Endocrinology* 135, 1100-1108 (1994).], and GHS was supposed to bind to a receptor different than GHRH receptor. Actually, a gene for a receptor to which GHS is bound was cloned, and from the result of Northern analysis, it was found that GHS receptor (GHS-R) is expressed in hypothalamus and brain pituitary gland, and that there is 90 % or more homology between the amino acid sequences of porcine- and human-derived GHS receptors [A. D. Howard, et al., *Science* 273, 974-977 (1996)]. However, an endogenous ligand that binds to GHS-R has not been isolated, and this GHS-R was an orphan receptor whose ligand was not evident.

**[0010]** In some cases, fatty acids such as myristic acid, geranic acid, palmitoyl acid or farnesyl acid are bound to the amino-terminal of a certain protein or to side chains of its amino acid residues, and the role of these fatty acids is anchoring such fatty acid-modified protein to cell membrane [J. Kendrew, et al., Eds., *The Encyclopedia of Molecular Biology* (Blackwell Science Ltd., London, 1994), p.616]. In such fatty acid-modified protein, the fatty acid binds to a cysteine residue via S-acyl linkage, and neither an amino acid having fatty acid bound to serine residue via O-acyl linkage, such as the endogenous GHS disclosed in the present invention, nor protein or peptide containing such fatty acid-modified amino acid, is known. Neither is it known for which the peptide containing such fatty acid-modified amino acid functions as a ligand for any receptor.

#### Disclosure of Invention

**[0011]** Before the present invention is described in detail, terms are defined as follows:

The term "peptide" refers to a compound comprising a plurality of amino acids linked therein via peptide linkages. Here, the amino acid (also called an amino acid residue) includes naturally occurring amino acids represented by formula:  $\text{NH}_2\text{-CH(R')-COOH}$ , wherein R' is a naturally occurring substituent group, as well as its D, L-optical isomers etc.

**[0012]** There is also a peptide, wherein a certain naturally occurring amino acid is replaced by a modified amino acid (also called a modified amino acid residue). The modified amino acid includes the amino acids of the above formula wherein the substituent group R' is further modified, its D, L-optical isomers thereof, and non-natural amino acids wherein e.g. various substituent groups are bound to the substituent group R' of the above formula via or not via an ester, ether, thioester, thioether, amide, carbamide or thiocarbamide linkage. The modified amino acid also includes non-natural amino acids whose amino groups are replaced by lower alkyl groups.

**[0013]** The terms "peptide analogue" refer to a compound wherein at least one amino acid in a peptide is replaced by a non-amino acid compound, and thus at least one linkage of said substituent compound to the peptide analogue

is not a peptide linkage.

**[0014]** Further, those compounds derived from these peptides and peptide analogues by modifying the amino-terminal and/or carboxyl-terminal thereof are referred to as derivatives. And the peptides, peptide analogues and derivatives thereof are referred collectively to as "peptide-type compound".

5 **[0015]** In the amino acid sequence set forth in SEQ ID NO: 2, an amino acid sequence of the 1st to 4th amino acids refers to Gly Ser Ser Phe,

an amino acid sequence of the 1st to 5th amino acids refers to Gly Ser Ser Phe Leu,

an amino acid sequence of the 1st to 6th amino acids refers to Gly Ser Ser Phe Leu Ser,

10 an amino acid sequence of the 1st to 7th amino acids refers to Gly Ser Ser Phe Leu Ser Pro,

an amino acid sequence of the 1st to 8th amino acids refers to Gly Ser Ser Phe Leu Ser Pro Glu,

an amino acid sequence of the 1st to 9th amino acids refers to Gly Ser Ser Phe Leu Ser Pro Glu His, and

an amino acid sequence of the 1st to 10th amino acids refers to Gly Ser Ser Phe Leu Ser Pro Glu His Gln.

**[0016]** The discovery of an endogenous ligand (endogenous GHS) which binds to GHS receptor to exhibit an activity for increasing the intracellular calcium ion concentration or for inducing GH secretion has been desired together with  
15 a method of utilizing the same. Further, a compound has been desired, which is a structural analogue of said endogenous GHS and has an activity for increasing the intracellular calcium ion concentration or for inducing GH secretion. Further, a pharmaceutical composition or a composition for promoting animal growth has been desired, which comprises said endogenous GHS or its structural analogous inducing pulsatile GH secretion thereby eliminating side effects by GH administration, as well as a therapeutic application using said composition.

20 **[0017]** The present inventors focused their attention on the fact that binding of the ligand to GHS receptor (GHS-R) causes a transient increase in the intracellular calcium ion concentration with inositol phospholipid as second messenger, and they screened extracts of various organs and tissues by using the activity of increasing the intracellular calcium ion concentration (Ca-releasing activity) as an indicator in CHO cells (CHO-GHSR62) expressing GHS-R. As a result, the inventors found that stomach extracts of rat has a strong Ca-releasing activity, and successfully purified a substance  
25 having a strong Ca-releasing activity from the above extracts by various kinds of chromatography, and found that said substance is a novel peptide modified with fatty acid, having a molecular weight of about 3,000. Further, they confirmed that said novel peptide promotes specific secretion of GH from cells of anterior pituitary, and found that said novel peptide is an endogenous ligand for GHS-R, that is, an endogenous GH secretagogue (endogenous GHS). That is, the first aspect of the present invention is directed to an endogenous GH secretion-inducing peptide having the activity  
30 of increasing the intracellular calcium ion concentration or the activity of inducing GH secretion, wherein a certain constituent amino acid residue is modified with fatty acid, as well as a method for preparing said peptide.

**[0018]** The present inventors precisely analyzed the structure of the endogenous GH secretion-inducing peptide, and found that said peptide is a peptide consisting of the amino acid sequence set forth in SEQ ID NO: 2, wherein the side-chain hydroxyl group of 3rd serine from the Amino-terminal has been acylated with fatty acid. Further, a human-  
35 derived GH secretion-inducing peptide was also purified from human stomach extract having a strong Ca-releasing activity similar to that of rat stomach extract and analyzed for its structure in the same manner as for rat-derived GH secretion-inducing peptide, and as a result the inventors found that the human-derived endogenous GH secretion-inducing peptide consists of the amino acid sequence set forth in SEQ ID NO: 3, wherein the side-chain hydroxyl group of 3rd serine from the amino-terminal has been acylated with fatty acid. Comparison between the amino acid sequences  
40 of the rat- and human-derived endogenous GH secretion-inducing peptides revealed homology as high as 89 % as a whole.

**[0019]** Specifically, the rat- and human-derived peptides are identical in an amino acid sequence of the 1st to 10th amino acids from amino-terminal and in an amino acid sequence of the 13th to 28th amino acids from amino-terminal, but are different in the 11th to 12th amino acids which are lysine and alanine in the rat peptide, which are replaced by  
45 arginine and valine in the human peptide, respectively. The rat-derived endogenous GH secretion-inducing peptide was cleaved with various proteases and its purified peptide fragments were measured for Ca-releasing activity, and as a result, a peptide consisting of the 1st to 7th amino acids from amino-terminal was the minimum peptide having the Ca-releasing activity.

**[0020]** By measurement of the Ca-releasing activity of chemically synthesized peptides, the inventors found that the core sequence essential for eliciting the Ca-releasing activity is a sequence consisting of 4 amino acids set forth in  
50 SEQ ID NO: 8. Further, the sequence consisting of 10 amino acids set forth in SEQ ID NO: 9 was conserved in non-rat endogenous GH secretion-inducing peptides (each consisting of 28 amino acids) separated from human, porcine and bovine, as well as in endogenous GH secretion-inducing peptides (each consisting of 27 amino acids) wherein one glutamine was deleted from the above peptides.

55 **[0021]** That is, the second aspect of the present invention is directed to a fatty acid-modified peptide comprising the amino acid sequence set forth in SEQ ID NO: 8, preferably the amino acid sequence set forth in SEQ ID NO: 1 and more preferably the amino acid sequence set forth in SEQ ID NO: 9 as the core sequence essential for eliciting the Ca-releasing activity.

[0022] Endogenous GH secretion-inducing peptides were also isolated from chicken, eel and frog, and these peptides were found to have a core sequence consisting of 4 amino acids set forth in SEQ ID NO: 8.

[0023] In addition, an endogenous GH secretion-inducing peptide very similar to the rat endogenous GH secretion-inducing peptide was also isolated from frog.

5 [0024] Further, endogenous GH secretion-inducing peptides were also isolated from *Xenopus laevis*, rainbow trout (*Oncorhynchus mykiss*), and dog. From rainbow trout, ghrelin-23 consisting of 23 amino acids and ghrelin-20 consisting of 20 amino acids were isolated respectively.

[0025] The carboxyl-terminal amino acid of eel ghrelin, rainbow trout ghrelin-23 and ghrelin-20 was amidated.

10 [0026] Because an amino acid residue at the 3rd position from the amino-terminal in the endogenous GH secretion-inducing peptide from *Xenopus laevis* is threonine, the present invention also relates to a fatty acid-modified peptide, which contains, as the core sequence essential for exhibiting the Ca-releasing activity, a peptide wherein the amino acid residue 3rd serine was replaced by threonine in the amino acid sequence set forth in SEQ ID NO: 8, preferably the amino acid sequence set forth in SEQ ID NO: 1 and more preferably the amino acid sequence set forth in SEQ ID NO: 9.

15 [0027] The endogenous fatty acid-modified peptide having GH secretion-inducing activity or the fatty acid-modified peptide consisting of said core sequence, disclosed in the present invention, also provides a guideline for designing a compound having Ca-releasing activity.

[0028] That is, in the third aspect of the present invention, a novel compound having Ca-releasing activity is obtained by synthesizing a structural analogue of said fatty acid-modified peptide by confirming the Ca-releasing activity of the resulting structural analogue. Accordingly, the present invention also encompasses a peptide or peptide analogue having the activity of increasing the intracellular calcium ion concentration, wherein a certain constituent amino acid is replaced by a modified amino acid or non-amino acid compound.

20 [0029] A cDNA coding the endogenous GH secretion-inducing peptide was obtained in a usual manner. Each of rat and human cDNAs consists of 117 amino acids as shown in the amino acid sequences in SEQ ID NOS: 4 and 5, and the amino acid sequences of rat and human endogenous GH secretion-inducing peptides were identical in a sequence of 28 amino acids from the 24th to 51st positions from the amino-terminal, respectively. That is, it was revealed that the endogenous GH secretion-inducing peptide is synthesized as a precursor peptide consisting of 117 amino acids, then a signal peptide consisting of amino-terminal 23 amino acids is cleaved off and further carboxyl-terminal 56 amino acids are cleaved off, whereby the fatty acid-modified peptide having GH secretion-inducing activity is formed. In addition, a cDNA coding a precursor of the endogenous GH secretion-inducing peptide consisting of 28 amino acids was also found in porcine.

[0030] Further, a cDNA coding a precursor for the endogenous GH secretion-inducing peptide consisting of 27 amino acids was found in porcine.

35 [0031] Further, a partial cDNA coding a precursor for the endogenous GH secretion-inducing peptide consisting of 27 amino acids was found in bovine.

[0032] Further, a cDNA coding a precursor of the endogenous GH secretion-inducing peptide was also found in eel, *Xenopus laevis* and rainbow trout. From rainbow trout, a cDNA coding a precursor of ghrelin-23 consisting of 23 amino acids and a cDNA coding a precursor of ghrelin-20 of 20 amino acids were isolated respectively.

40 [0033] Accordingly, the fourth aspect of the present invention lies in a cDNA coding a precursor of the endogenous GH secretion-inducing peptide, as well as a method for producing a peptide as a starting material of the fatty acid-modified peptide or peptide analogue having Ca-releasing activity, which comprises using said cDNA.

[0034] In purification of the endogenous GH secretion-inducing peptide (ghrelin) composed of 28 amino acids from rat stomach extract, a peptide recovered as a minor fraction was analyzed, and as a result a peptide consisting of 27 amino acids (ghrelin-27), which is a peptide of ghrelin from which 13th or 14th glutamine had been deleted, was found. 45 Ghrelin-27 has completely the same Ca-releasing activity and GH secretion-inducing activity as those of ghrelin consisting of 28 amino acids, and ghrelin-27 is an endogenous GH secretion-inducing peptide, and thus ghrelin-27 also falls under the scope of the present invention.

[0035] The nucleotide sequence coding 13th and 14th glutamine in ghrelin is gca gca, which is a terminal exon sequence to be subjected to mRNA splicing, thus suggesting the possibility of formation of a cDNA from which one of two codons for glutamine residues was deleted by different splicing. Actually, a cDNA coding a precursor peptide of ghrelin-27 consisting of 27 amino acids was found in screening of rat and human cDNA libraries.

50 [0036] That is, it was revealed that rat and human ghrelin-27 peptide is synthesized as a precursor peptide consisting of 116 amino acids set forth in SEQ ID NO: 12 or 13, then a signal peptide consisting of amino-terminal 23 amino acids is cleaved off and further carboxyl-terminal 56 amino acids are cleaved off, whereby a fatty acid-modified peptide consisting of 27 amino acids having GH secretion-inducing activity (ghrelin-27) is formed.

55 [0037] Further, a cDNA coding a precursor of ghrelin-27 peptide was found in porcine and bovine, and the presence of ghrelin-27 and its precursor was confirmed in these animals.

[0038] That is, the present invention also encompasses ghrelin-27 peptide consisting of an amino acid sequence

set forth in SEQ ID NOS: 10, 11, 17 and 22, a ghrelin-27 precursor peptide having an amino acid sequence set forth in SEQ ID NOS: 12, 13, 19 and 23, and a cDNA coding said precursor peptide which comprises a nucleotide sequence set forth in SEQ ID NOS: 14, 15, 21 and 24.

5 **[0039]** The fatty acid-modified peptide having Ca-releasing activity and the peptide-type compound such as peptide analogue having Ca-releasing activity as disclosed in the present invention also provide a pharmaceutical composition for treating diseases attributable to defect or decrease in GH. Said pharmaceutical composition can be used to treat any diseases against which the GH administration is effective, and various side effects caused by GH administration can be overcome. Further, said pharmaceutical composition can also be used as an animal drug such as a growth-promoting agent for animals.

10 **[0040]** Because the peptide-type compound of the present invention has an appetite-promoting action by administration into ventricle and intravenous administration, and thus it can be used as an appetite promoting agent for treating loss of appetite or sitophobia. In addition, the present peptide-type compound has a stomach motility- and gastric acid secretion-promoting action, and thus it can also be used as an agent for treating stomach functional diseases such as non-ulcer indigestion, sudden light stomach atony, functional indigestion, and reflux esophagitis. Further, the present  
15 peptide-type compound exhibits a cell growth-promoting action in bone marrow, duodenum and jejunum by intravenous administration, and thus it can be used as an agent for protecting mucous membrane on intestinal tract, an agent for preventing damage to mucous membrane on small intestine during intravenous nutrition and an agent for treating osteoporosis.

20 **[0041]** The present invention also encompasses an antibody prepared by using the fatty acid-modified peptide having Ca-releasing activity disclosed in the present invention as an antigen, a method for measuring the endogenous GH secretion-inducing peptide by use of said antibody, and a measurement kit comprising said antibody.

**[0042]** Further, the present invention encompasses an assay method for separating and quantifying ghrelin modified with a fatty acid and ghrelin from which the fatty acid was eliminated, which comprises using two antibodies built up to N- and carboxyl-terminal peptides from ghrelin, the former antibody capable of recognizing fatty acid-modified 3rd  
25 serine, as well as an assay kit comprising a combination of the antibodies against N- and carboxyl-terminal peptides from ghrelin.

**[0043]** That is, the present invention provides a novel peptide hormone having a novel modified amino acid i.e. acylated serine, and also provides a guideline for novel design of a compound having Ca-releasing activity with the structure of said peptide as a fundamental skeleton.

30 **[0044]** Further, the elucidation of the mechanism of induction of GH secretion by the fatty acid-modified peptide disclosed in the present invention or by GH releasing hormone and somatostatin is suggested to be extendable not only to the mechanism of induction of GH secretion but also to the mechanism of regulating secretion of other hormones. The present invention discloses various functions of the fatty acid-modified peptide as a regulatory factor in the circulatory system and the metabolic system, and the effect of the present invention extends to the elucidation of a new  
35 biological regulatory mechanism.

**[0045]** Specifically, the present invention relates to:

(1) A peptide-type compound, wherein in a peptide having the activity of increasing the intracellular calcium ion concentration, at least one amino acid is replaced by a modified amino acid and/or a non-amino acid compound,  
40 or a pharmaceutically acceptable salt thereof;

(2) The peptide-type compound according to item (1), which comprises (a) an amino acid sequence set forth in SEQ ID NO: 2 or (b) an amino acid sequence having any one of amino acid sequences selected from the group consisting of

- 45 (1) amino acid sequence of amino acids 1 to 4,  
(2) amino acid sequence of amino acids 1 to 5,  
(3) amino acid sequence of amino acids 1 to 6,  
(4) amino acid sequence of amino acids 1 to 7,  
(5) amino acid sequence of amino acids 1 to 8,  
50 (6) amino acid sequence of amino acids 1 to 9, and  
(7) amino acid sequence of amino acids 1 to 10

from the amino-terminal in the sequence (a) and at least one amino acid deleted, replaced and/or added in a part outside said amino acid sequences, or a pharmaceutically acceptable salt thereof;

55 (3) The peptide-type compound according to item (2) above, which comprises one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 3, 4, 5, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, 22 and 23, or a pharmaceutically acceptable salt thereof;

(4) The peptide-type compound according to item (2) above, which comprises one amino acid sequence selected

from the group consisting of amino acid sequences set forth in SEQ ID NOS: 25, 26, 29, 30, 31, 32, 34 and 35, or a pharmaceutically acceptable salt thereof;

(5) A peptide-type compound, wherein in a peptide having the activity of increasing the intracellular calcium ion concentration and the activity of inducing secretion of growth hormone, (a) constitutional amino acids are modified or not modified and (b) at least one amino acid is replaced or not replaced by a non-amino acid compound, or a pharmaceutically acceptable salt thereof;

(6) The peptide-type compound according to item (1) or (5) which comprises amino acid sequences set forth in SEQ ID NOS: 27, 28 and 33, or a pharmaceutically acceptable salt thereof;

(7) The peptide-type compound according to item (5), which comprises (a) an amino acid sequence set forth in SEQ ID NO: 2 or (b) an amino acid sequence having any one of amino acid sequences selected from the group consisting of

- (1) amino acid sequence of amino acids 1 to 4,
- (2) amino acid sequence of amino acids 1 to 5,
- (3) amino acid sequence of amino acids 1 to 6,
- (4) amino acid sequence of amino acids 1 to 7,
- (5) amino acid sequence of amino acids 1 to 8,
- (6) amino acid sequence of amino acids 1 to 9, and
- (7) amino acid sequence of amino acids 1 to 10

from the amino-terminal in the sequence (a) and at least one amino acid deleted, replaced and/or added in a part outside said amino acid sequences, or a pharmaceutically acceptable salt thereof;

(8) The peptide-type compound according to item (7) above, which comprises one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 3, 4, 5, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, 22 and 23, or a pharmaceutically acceptable salt thereof;

(9) The peptide-type compound according to item (7) above, which comprises one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 25, 26, 29, 30, 31, 32, 34 and 35, or a pharmaceutically acceptable salt thereof;

(10) The peptide-type compound according to item (1) or (5) above, whose amino-terminal amino acids 1 to 4 are represented by formula:



wherein the symbol A is either an amino acid or a non-amino acid compound, or is missing, and the symbol B is either an amino acid or a non-amino acid compound, or is missing, provided that the length of the A + B molecular chain is a dipeptide length, and the symbol C or the symbol D may be the same or different and represents (a) a modified amino acid, (b) an amino acid having a hydrophobic residue, or (c) an amino acid having a basic side chain, or a pharmaceutically acceptable salt thereof;

(11) The peptide-type compound according to item (10), wherein the symbol C is a modified amino acid in which (a) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid via or not via an alkylene group containing one or more carbon atoms and via an ester, ether, thioether, amide or disulfide linkage, or (b) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid, and the symbol D is an amino acid having a hydrophobic residue, or a pharmaceutically acceptable salt thereof;

(12) A peptide-type compound, wherein in one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 2, 3, 9, 10, 11, 16, 17, 22, 25, 26, 27, 28, 29, 30 and 31, an amino acid sequence of amino-terminal amino acids 1 to 4 is replaced by the structure of the peptide-type compound described in item (10) or (11), or a pharmaceutically acceptable salt thereof;

(13) The peptide-type compound according to item (1), (2), (3), (5), (7) or (8) above, wherein the modified amino acid is an amino acid at the 3rd position from the amino-terminal, or a pharmaceutically acceptable salt thereof;

(14) The peptide-type compound according to item (13), wherein the amino acid in the modified amino acid is serine or cysteine, or a pharmaceutically acceptable salt thereof;

(15) The peptide-type compound according to item (1), (2), (3), (5), (7) or (8) above, which comprises a modified amino acid in which (a) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid via or not via an alkylene group containing one or more carbon atoms and via an ester, ether, thioester, thioether, amide or carbamide linkage, or (b) H or a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid, or a pharma-

ceutically acceptable salt thereof;

(16) The peptide-type compound according to item (1), (2), (4), (5), (6), (7), (9), (10) or (12) above, wherein the modified amino acid is an amino acid in which (a) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom via or not via an alkylene group containing one or more carbon atoms and via an ester, ether, thioester, thioether, disulfide, amide, carbamide or thiocarbamide linkage, or (b) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon, or a pharmaceutically acceptable salt thereof;

(17) The peptide-type compound according to item (1), (2), (3), (5), (7) or (8) above, which comprises a modified amino acid modified with an ester linkage, or a pharmaceutically acceptable salt thereof;

(18) The peptide-type compound according to item (1), (2), (4), (5), (6), (7), (9), (10), (11) or (12) above, which comprises a modified amino acid modified by conversion of a functional group in a side chain of said amino acid into an ester linkage, or a pharmaceutically acceptable salt thereof;

(19) The peptide-type compound according to item (17) above, which comprises an amino acid having a fatty acid bound via an ester linkage to a side-chain hydroxyl group of said amino acid, or a pharmaceutically acceptable salt thereof;

(20) The peptide-type compound according to item (18) above, which comprises an amino acid having a fatty acid bound via an ester linkage to a side-chain hydroxyl group of said amino acid or via a thioester linkage to a side-chain mercapto group of said amino acid, or a pharmaceutically acceptable salt thereof;

(21) The peptide-type compound according to item (19) above, which comprises an amino acid to which a fatty acid containing 2 to 35 carbon atoms was bound, or a pharmaceutically acceptable salt thereof;

(22) The peptide-type compound according to item (20) above, wherein the fatty acid contains 2 to 35 carbon atoms, or a pharmaceutically acceptable salt thereof;

(23) The peptide-type compound according to item (21) above, which comprises an amino acid to which a fatty acid selected from the group consisting of fatty acids containing 2, 4, 6, 8, 10, 12, 14, 16 and 18 carbon atoms was bound, or a pharmaceutically acceptable salt thereof;

(24) The peptide-type compound according to item (22) above, wherein the fatty acid is a fatty acid selected from the group consisting of fatty acids containing 2, 4, 6, 8, 10, 12, 14, 16 and 18 carbon atoms, or a pharmaceutically acceptable salt thereof;

(25) The peptide-type compound according to item (23) above, wherein the bound fatty acid is octanoic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof;

(26) The peptide-type compound according to item (24) above, wherein the fatty acid is octanoic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof;

(27) The peptide-type compound according to item (23) above, wherein the bound fatty acid is decanoic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof;

(28) The peptide-type compound according to item (24) above, wherein the fatty acid is decanoic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof;

(29) A peptide-type compound comprising a basic amino acid bound to the carboxyl-terminal of a peptide-type compound described in items (1) to (28) above;

(30) The peptide-type compound according to items (1), (2), (3), (5), (7), (8), (13), (14), (15), (17), (19), (21), (23), (25) and (27) above, wherein the amino-terminal is modified with a saturated or unsaturated alkyl or acyl group containing one or more carbon atoms, and/or a hydroxyl group of the carboxyl-terminal carboxyl group is OZ or NR<sub>2</sub>R<sub>3</sub> wherein Z is a pharmaceutically acceptable cation or a lower branched or linear alkyl group, and R<sub>2</sub> and R<sub>3</sub> are the same or different and represent H or a lower branched or linear alkyl group;

(31) The peptide-type compound according to items (1), (2), (4), (5), (6), (7), (9), (10), (11), (12), (16), (18), (20), (22), (24), (26), (28) and (29) above, wherein the amino-terminal amino group is modified by introduction of a saturated or unsaturated alkyl or acyl group containing one or more carbon atoms, and/or a hydroxyl group of the carboxyl-terminal carboxyl group is OZ or NR<sub>2</sub>R<sub>3</sub> wherein Z is a pharmaceutically acceptable cation or a lower branched or linear alkyl group, and R<sub>2</sub> and R<sub>3</sub> are the same or different and represent H or a lower branched or linear alkyl group;

(32) A peptide-type compound comprising a basic group introduced into a carboxyl-terminal amide derivative of a peptide-type compound described in item (30) or (31) above;

(33) A pharmaceutical composition comprising a peptide-type compound described in items (1) to (32) above or a pharmaceutically acceptable salt thereof as an active ingredient;

(34) A pharmaceutical composition for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises a peptide-type compound described in items (1) to (32) above or a pharmaceutically acceptable salt thereof as an active ingredient;

(35) A pharmaceutical composition for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises an agent for treating diseases not attributable to a defect or decrease in growth hormone



and a peptide-type compound described in items (1) to (32) above or a pharmaceutically acceptable salt thereof;  
 (36) A pharmaceutical composition according to items (33) to (35) above, which is applied to animals other than human beings;

5 (37) A method for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises administering a pharmaceutical composition comprising a peptide-type compound described in items (1) to (32) above or a pharmaceutically acceptable salt thereof as an active ingredient;

(38) A method for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises administering an agent for treating diseases not attributable to a defect or decrease in growth hormone and a peptide-type compound described in items (1) to (32) above or a pharmaceutically acceptable salt thereof;

10 (39) The treatment method according to items (37) or (38), which is applied to animals other than human beings;

(40) A DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (32) above, which comprises a nucleotide sequence coding a peptide containing an amino acid sequence recognizing at least one modifiable amino acid in the amino acid sequence encoded by said DNA;

15 (41) The DNA according to item (40) above, wherein the nucleotide sequence is one nucleotide sequence selected from the group consisting of nucleotide sequences set forth in SEQ ID NOS: 6, 7, 14, 15, 20, 21, 24, 36, 37, 38 and 39;

(42) The DNA according to item (40) above, wherein the nucleotide sequence is an amino acid-coding nucleotide sequence in one nucleotide sequence selected from the group consisting of nucleotide sequences set forth in SEQ ID NOS: 6, 7, 14, 15, 20, 21, 24, 36, 37, 38 and 39;

20 (43) A vector comprising a DNA described in items (40) to (42) above;

(44) Cells comprising the vector described in item (43) above;

(45) Cells comprising a DNA described in items (40) to (42) above, wherein a peptide-type compound having an amino acid sequence encoded by said DNA can be produced as a peptide-type compound having at least one amino acid modified in said amino acid sequence;

25 (46) An antibody against a peptide-type compound described in items (1) to (32) above;

(47) A method for assaying a peptide-type compound described in items (1) to (32) above, which comprises using the antibody described in item (46) above to detect the peptide-type compound described in items (1) to (32) above;

(48) A kit for detecting a peptide-type compound described in items (1) to (32) above, which comprises using the antibody described in item (46) above to detect the peptide-type compound described in items (1) to (32) above;

30 (49) A method for producing a peptide-type compound described in items (1) to (32) above by genetic recombination technology, which comprises transforming a vector containing a DNA described in items (40) to (42) above into host cells capable of modifying a side chain of at least one amino acid in said peptide, then culturing the resulting transformed cells and recovering the desired peptide-type compound from the culture;

35 (50) A method for producing a peptide-type compound described in items (1) to (32) above by genetic recombination technology, which comprises transforming a vector containing a DNA described in items (40) to (42) above into host cells, then culturing the resulting transformed cells and recovering the desired peptide-type compound from the culture, followed by chemically modifying an arbitrary amino acid thereof;

40 (51) A method for producing a peptide-type compound described in items (19) to (28) above by genetic recombination technology, which comprises using cells having the activity of binding a fatty acid via an ester linkage to a side-chain hydroxyl group of an amino acid or via a thioester linkage to a side-chain mercapto group of an amino acid in the peptide-type compound;

(52) A method for producing a peptide-type compound described in items (19) to (28) above, which comprises using cells having the serine acylation activity of binding a fatty acid via an ester linkage to a side-chain hydroxyl group of serine in the amino acid sequence set forth in SEQ ID NO: 8;

45 (53) A process for producing a peptide-type compound described in items (19) to (28) above, which comprises using cells having the acylation activity of binding a fatty acid via an ester linkage to a side-chain hydroxyl group of threonine in the amino acid sequence set forth in SEQ ID NO: 28;

50 (54) A pharmaceutical composition for gene therapy for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (32) above into cells in a living body and expressing a peptide with at least one modified amino acid, the peptide having the activity of increasing the intracellular calcium ion concentration;

55 (55) A method for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (32) above into cells in a living body enabling a peptide having an amino acid sequence encoded by said DNA to be produced as a peptide having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence, whereby a peptide having the activity of inducing growth hormone is expressed;

(56) A pharmaceutical composition for gene therapy for treatment of diseases not attributable to a defect or de-

crease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (32) above into cells in a living body and expressing a peptide with at least one modified amino acid, the peptide having the activity of increasing the intracellular calcium ion concentration;

5 (57) A method for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (32) above into cells in a living body enabling a peptide having an amino acid sequence encoded by said DNA to be produced as a peptide having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence, whereby a peptide having the activity of inducing growth hormone is expressed.

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[0046] Specifically, the present invention also relates to:

(1) A peptide-type compound, wherein in a peptide having the activity of increasing the intracellular calcium ion concentration, at least one amino acid is replaced by a modified amino acid and/or a non-amino acid compound, or a pharmaceutically acceptable salt thereof;

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(2) The peptide-type compound according to item (1) above, which comprises (a) an amino acid sequence set forth in SEQ ID NO: 2 or (b) an amino acid sequence having any one of amino acid sequences selected from the group consisting of

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- (1) amino acid sequence of amino acids 1 to 4,
- (2) amino acid sequence of amino acids 1 to 5,
- (3) amino acid sequence of amino acids 1 to 6,
- (4) amino acid sequence of amino acids 1 to 7,
- (5) amino acid sequence of amino acids 1 to 8,
- 25 (6) amino acid sequence of amino acids 1 to 9, and
- (7) amino acid sequence of amino acids 1 to 10

25

from the amino-terminal in the sequence (a) and at least one amino acid deleted, replaced and/or added in a part outside said amino acid sequences, or a pharmaceutically acceptable salt thereof;

30

(3) The peptide-type compound according to item (2) above, which comprises one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 3, 4, 5, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, 22, 23, 25 and 26, or a pharmaceutically acceptable salt thereof;

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(4) A peptide-type compound wherein in a peptide having the activity of increasing the intracellular calcium ion concentration and the activity of inducing secretion of growth hormone, (a) constitutional amino acids are modified or not modified and (b) at least one amino acid is replaced or not replaced by a non-amino acid compound, or a pharmaceutically acceptable salt thereof;

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(5) The peptide-type compound according to item (1) or (4) which comprises an amino acid sequence set forth in SEQ ID NO: 27, or a pharmaceutically acceptable salt thereof;

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(6) The peptide-type compound according to item (4) above, which comprises (a) an amino acid sequence set forth in SEQ ID NO: 2 or (b) an amino acid sequence having any one of amino acid sequences selected from the group consisting of

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- (1) amino acid sequence of amino acids 1 to 4,
- (2) amino acid sequence of amino acids 1 to 5,
- (3) amino acid sequence of amino acids 1 to 6,
- (4) amino acid sequence of amino acids 1 to 7,
- (5) amino acid sequence of amino acids 1 to 8,
- (6) amino acid sequence of amino acids 1 to 9, and
- (7) amino acid sequence of amino acids 1 to 10

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from the amino-terminal in the sequence (a) and at least one amino acid deleted, replaced and/or added in a part outside said amino acid sequences, or a pharmaceutically acceptable salt thereof;

55

(7) The peptide-type compound according to item (6) above, which comprises one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 3, 4, 5, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, 22, 23, 25 and 26, or a pharmaceutically acceptable salt thereof;

55

(8) The peptide-type compound according to item (1) or (4) above, whose amino-terminal amino acids 1 to 4 are represented by formula:

## A-B-C-D-

wherein the symbol A is either an amino acid or a non-amino acid compound, or is missing, and the symbol B is either an amino acid or a non-amino acid compound, or is missing, provided that the length of the A + B molecular chain is a dipeptide length, and the symbol C or the symbol D may be the same or different and represents (a) a modified amino acid, (b) an amino acid having a hydrophobic residue or (c) an amino acid having a basic side chain, or a pharmaceutically acceptable salt thereof;

(9) A peptide-type compound, wherein in one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 2, 3, 8, 9, 10, 11, 16, 17, 22, 25 and 26, an amino acid sequence of amino-terminal amino acids 1 to 4 is replaced by the structure of the peptide-type compound described in item (8) above, or a pharmaceutically acceptable salt thereof.

(10) The peptide-type compound according to items (1) to (9) above, wherein the modified amino acid is an amino acid in which (a) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid via or not via an alkylene group containing one or more carbon atoms and via an ester, ether, thioester, thioether, disulfide, amide, carbamide or thiocarbamide linkage, or (b) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid, or a pharmaceutically acceptable salt thereof;

(11) The peptide-type compound according to items (1) to (10) above, which comprises a modified amino acid modified by conversion of a functional group in a side chain of said amino acid into an ester linkage, or a pharmaceutically acceptable salt thereof;

(12) The peptide-type compound according to item (11) above, which comprises an amino acid having a fatty acid bound via an ester linkage to a side-chain hydroxyl group or mercapto group of said amino acid, or a pharmaceutically acceptable salt thereof;

(13) The peptide-type compound according to item (12) above, wherein the fatty acid contains 2 to 35 carbon atoms, or a pharmaceutically acceptable salt thereof;

(14) The peptide-type compound according to item (12) above, wherein the fatty acid is a fatty acid selected from the group consisting of fatty acids containing 2, 4, 6, 8, 10, 12, 14, 16 and 18 carbon atoms, or a pharmaceutically acceptable salt thereof;

(15) The peptide-type compound according to item (12) above, wherein the fatty acid is octanoic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof;

(16) The peptide-type compound according to item (12) above, wherein the fatty acid is decanoic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof;

(17) The peptide-type compound according to items (1) to (16) above, wherein the amino-terminal amino group is modified by introduction of a saturated or unsaturated alkyl or acyl group containing one or more carbon atoms, and/or a hydroxyl group of the carboxyl-terminal carboxyl group is OZ or NR<sub>2</sub>R<sub>3</sub> wherein Z is a pharmaceutically acceptable cation or a lower branched or linear alkyl group, and R<sub>2</sub> and R<sub>3</sub> are the same or different and represent H or a lower branched or linear alkyl group;

(18) A peptide-type compound comprising a basic amino acid bound to the carboxyl-terminal of a peptide-type compound described in items (1) to (16) above;

(19) A peptide-type compound comprising a basic group introduced into a carboxyl-terminal amide derivative of the peptide-type compound described in items (1) to (16) or (18) above;

(20) A pharmaceutical composition comprising a peptide-type compound described in items (1) to (19) above or a pharmaceutically acceptable salt thereof as an active ingredient;

(21) A pharmaceutical composition for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises a peptide-type compound described in items (1) to (19) above or a pharmaceutically acceptable salt thereof as an active ingredient;

(22) A pharmaceutical composition for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises an agent for treating diseases not attributable to a defect or decrease in growth hormone and a peptide-type compound described in items (1) to (19) above or a pharmaceutically acceptable salt thereof;

(23) A pharmaceutical composition according to items (20) to (22), which is applied to animals other than human beings;

(24) A method for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises administering a pharmaceutical composition comprising a peptide-type compound described in items (1) to (19) above or a pharmaceutically acceptable salt thereof as an active ingredient;

(25) A method for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises administering an agent for treating diseases not attributable to a defect or decrease in growth hormone and a peptide-type compound described in items (1) to (19) above or a pharmaceutically acceptable salt thereof;

- (26) A method according to item (24) or (25), which is applied to animals other than human beings;
- (27) A DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (19) above, which comprises a nucleotide sequence coding a peptide containing an amino acid sequence recognizing at least one modifiable amino acid in the amino acid sequence encoded by said DNA;
- 5 (28) The DNA according to item (27) above, wherein the nucleotide sequence is one nucleotide sequence selected from the group consisting of nucleotide sequences set forth in SEQ ID NOS: 6, 7, 14, 15, 20, 21 and 24;
- (29) The DNA according to item (27) above, wherein the nucleotide sequence is an amino acid-coding nucleotide sequence in one nucleotide sequence selected from the group consisting of nucleotide sequences set forth in SEQ ID NOS: 6, 7, 14, 15, 20, 21 and 24;
- 10 (30) A vector comprising a DNA described in items (27) to (29) above;
- (31) Cells comprising the vector described in item (30) above;
- (32) Cells comprising a DNA described in items (27) to (29) above, wherein a peptide-type compound having an amino acid sequence encoded by said DNA can be produced as a peptide-type compound having at least one amino acid modified in said amino acid sequence;
- 15 (33) An antibody against a peptide-type compound described in items (1) to (19) above;
- (34) A method for assaying a peptide-type compound described in items (1) to (19) above, which comprises using the antibody described in item (33) above to detect the peptide-type compound described in items (1) to (19) above;
- (35) A kit for detecting a peptide-type compound described in items (1) to (19) above, which comprises using the antibody described in item (33) above to detect the peptide-type compound described in items (1) to (19) above;
- 20 (36) A method for producing a peptide-type compound described in items (1) to (19) above by genetic recombination technology, which comprises transforming a vector containing a DNA described in items (27) to (29) above into host cells capable of modifying a side chain of at least one amino acid in said peptide, then culturing the resulting transformed cells and recovering the desired peptide-type compound from the culture;
- (37) A method for producing a peptide-type compound described in items (1) to (19) above by genetic recombination technology, which comprises transforming a vector containing a DNA described in items (27) to (29) above into host cells, then culturing the resulting transformed cells and recovering the desired compound from the culture, followed by chemically modifying an arbitrary amino acid thereof;
- 25 (38) A method for producing a peptide-type compound described in items (12) to (16) above by genetic recombination technology, which comprises using cells having the activity of binding a fatty acid via an ester linkage to a side-chain hydroxyl group or a side-chain mercapto group of an amino acid in the peptide-type compound;
- (39) A method for producing a peptide-type compound described in items (12) to (16) above, which comprises using cells having the serine acylation activity of binding a fatty acid via an ester linkage to a side-chain hydroxyl group of serine in the amino acid sequence set forth in SEQ ID NO: 8;
- 30 (40) A pharmaceutical composition for gene therapy for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (19) above into cells in a living body and expressing a peptide with at least one modified amino acid, the peptide having the activity of increasing the intracellular calcium ion concentration;
- 35 (41) A method for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (19) above into cells in a living body enabling a peptide having an amino acid sequence encoded by said DNA to be produced as a peptide having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence, whereby a peptide having the activity of inducing growth hormone is expressed;
- 40 (42) A pharmaceutical composition for gene therapy for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (19) above into cells in a living body and expressing a peptide with at least one modified amino acid, the peptide having the activity of increasing the intracellular calcium ion concentration;
- 45 (43) A method for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (19) above into cells in a living body enabling a peptide having an amino acid sequence encoded by said DNA to be produced as a peptide having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence, whereby a peptide having the activity of inducing growth hormone is expressed.

55 [0047] Specifically, the present invention also relates to:

- (1) A peptide-type compound having the activity of increasing the intracellular calcium ion concentration, wherein at least one amino acid is replaced by a modified amino acid and/or a non-amino acid compound, or a pharma-

ceutically acceptable salt thereof;

(2) The peptide-type compound according to item (1) above, which comprises (a) an amino acid sequence set forth in SEQ ID NO: 2 or (b) an amino acid sequence having any one of amino acid sequences selected from the group consisting of

- (1) amino acid sequence of amino acids 1 to 4,
- (2) amino acid sequence of amino acids 1 to 5,
- (3) amino acid sequence of amino acids 1 to 6,
- (4) amino acid sequence of amino acids 1 to 7,
- (5) amino acid sequence of amino acids 1 to 8,
- (6) amino acid sequence of amino acids 1 to 9, and
- (7) amino acid sequence of amino acids 1 to 10 from the amino-terminal in the sequence (a) and at least one amino acid deleted, replaced and/or added in a part outside said amino acid sequences, or a pharmaceutically acceptable salt thereof;

(3) The peptide-type compound according to item (2) above, which comprises one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 3, 4, 5, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, 22 and 23, or a pharmaceutically acceptable salt thereof;

(4) A peptide-type compound having the activity of increasing the intracellular calcium ion concentration and the activity of inducing secretion of growth hormone, wherein (a) constitutional amino acids are modified or not modified and (b) at least one amino acid is replaced or not replaced by a non-amino acid compound, or a pharmaceutically acceptable salt thereof;

(5) The peptide-type compound according to item (4) above, which comprises (a) an amino acid sequence set forth in SEQ ID NO: 2 or (b) an amino acid sequence having any one of amino acid sequences selected from the group consisting of

- (1) amino acid sequence of amino acids 1 to 4,
- (2) amino acid sequence of amino acids 1 to 5,
- (3) amino acid sequence of amino acids 1 to 6,
- (4) amino acid sequence of amino acids 1 to 7,
- (5) amino acid sequence of amino acids 1 to 8,
- (6) amino acid sequence of amino acids 1 to 9, and
- (7) amino acid sequence of amino acids 1 to 10

from the amino-terminal in the sequence (a) and at least one amino acid deleted, replaced and/or added in a part outside said amino acid sequences, or a pharmaceutically acceptable salt thereof;

(6) The peptide-type compound according to items (4) or (5) above, which comprises one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 3, 4, 5, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, 22 and 23, or a pharmaceutically acceptable salt thereof;

(7) The peptide-type compound according to items (1) to (6) above, wherein the modified amino acid is an amino acid at the 3rd position from the amino-terminal thereof, or a pharmaceutically acceptable salt thereof;

(8) The peptide-type compound according to item (7) above, wherein the amino acid in the modified amino acid is serine or cysteine, or a pharmaceutically acceptable salt thereof;

(9) The peptide-type compound according to items (1) to (6) above, which comprises a modified amino acid in which (a) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid via or not via an alkylene group containing one or more carbon atoms and via an ester, ether, thioester, thioether, amide or carbamide linkage, or (b) H or a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid, or a pharmaceutically acceptable salt thereof;

(10) The peptide-type compound according to items (1) to (6) above, which comprises a modified amino acid modified with an ester linkage, or a pharmaceutically acceptable salt thereof;

(11) The peptide-type compound according to item (10) above, which comprises an amino acid to which a fatty acid was bound, or a pharmaceutically acceptable salt thereof;

(12) The peptide-type compound according to item (11) above, which comprises an amino acid to which a fatty acid containing 2 to 35 carbon atoms was bound, or a pharmaceutically acceptable salt thereof;

(13) The peptide-type compound according to item (12) above, which comprises an amino acid to which a fatty acid selected from the group of consisting fatty acids containing 2, 4, 6, 8, 10, 12, 14, 16 and 18 carbon atoms was bound, or a pharmaceutically acceptable salt thereof;

- (14) The peptide-type compound according to item (13) above, wherein the bound fatty acid is octanoic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof;
- (15) The peptide-type compound according to item (13) above, wherein the bound fatty acid is decanoic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof;
- 5 (16) The peptide-type compound according to items (1) to (15) above, wherein the amino-terminal is modified with a saturated or unsaturated alkyl or acyl group containing one or more carbon atoms, and/or a hydroxyl group of the carboxyl-terminal carboxyl group is OZ or NR<sub>2</sub>R<sub>3</sub> wherein Z is a pharmaceutically acceptable cation or a lower branched or linear alkyl group, and R<sub>2</sub> and R<sub>3</sub> are the same or different and represent H or a lower branched or linear alkyl group;
- 10 (17) A pharmaceutical composition comprising a peptide-type compound described in items (1) to (16) above or a pharmaceutically acceptable salt thereof as an active ingredient;
- (18) A pharmaceutical composition for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises a peptide-type compound described in items (1) to (16) above or a pharmaceutically acceptable salt thereof as an active ingredient;
- 15 (19) A pharmaceutical composition for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises an agent for treating diseases not attributable to a defect or decrease in growth hormone and a peptide-type compound described in items (1) to (16) above or a pharmaceutically acceptable salt thereof;
- (20) A pharmaceutical composition according to items (17) to (19), which is applied to animals other than human beings;
- 20 (21) A method for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises administering a pharmaceutical composition comprising a peptide-type compound described in items (1) to (16) above or a pharmaceutically acceptable salt thereof as an active ingredient;
- (22) A method for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises administering an agent for treating diseases not attributable to a defect or decrease in growth hormone and a peptide-type compound described in items (1) to (16) above or a pharmaceutically acceptable salt thereof;
- 25 (23) A method for treatment according to items (21) to (22), which is applied to animals other than human beings;
- (24) A DNA for a peptide-type compound described in items (1) to (16) above, which comprises a nucleotide sequence coding a peptide containing an amino acid sequence recognizing at least one modifiable amino acid in the amino acid sequence encoded by said DNA;
- 30 (25) The DNA according to item (24) above, which comprises one nucleotide sequence selected from the group consisting of nucleotide sequences set forth in SEQ ID NOS: 6, 7, 14, 15, 20, 21 and 24;
- (26) The DNA according to item (24) above, which comprises an amino acid-coding nucleotide sequence in one nucleotide sequence selected from the group consisting of nucleotide sequences set forth in SEQ ID NOS: 6, 7, 14, 15, 20, 21 and 24;
- 35 (27) A vector comprising a DNA described in items (24) to (26) above;
- (28) Cells comprising the vector described in item (27) above;
- (29) Cells comprising a vector containing a DNA described in items (24) to (26) above, wherein a peptide-type compound having an amino acid sequence encoded by said DNA can be produced as a peptide-type compound having at least one amino acid modified in said amino acid sequence;
- 40 (30) An antibody against a peptide-type compound described in items (1) to (16) above;
- (31) A method for assaying a peptide-type compound described in items (1) to (16) above, which comprises using the antibody described in item (30) above to detect the peptide-type compound described in items (1) to (16) above;
- (32) A kit for detecting a peptide-type compound described in items (1) to (16) above, which comprises using the antibody described in item (30) above to detect said peptide-type compound;
- 45 (33) A method for producing a peptide-type compound described in items (1) to (16) above by genetic recombination technology, which comprises transforming a vector containing a DNA described in items (24) to (26) above into host cells capable of modifying a side chain of at least one amino acid in said peptide, then culturing the resulting transformed cells and recovering the desired peptide-type compound from the culture;
- (34) A method for producing a peptide-type compound described in items (1) to (16) above by genetic recombination technology, which comprises transforming a vector containing a DNA described in items (24) to (26) above into host cells, then culturing the resulting transformed cells and recovering the desired peptide-type compound from the culture, followed by chemically modifying an arbitrary amino acid thereof;
- 50 (35) A method for producing the peptide-type compound described in items (11) to (15) above by genetic recombination technology, wherein the peptide-type compound can be produced as a peptide having a fatty acid bound to a serine residue in the amino acid sequence set forth in SEQ ID NO: 8;
- 55 (36) A method for producing a peptide-type compound having the activity of increasing the intracellular calcium ion concentration and the activity of inducing secretion of growth hormone, which comprises transforming a vector containing a DNA coding a peptide-type compound described in items (4) to (6) above into host cells, and culturing

the resulting transformed cells and recovering the desired compound from the culture;

(37) A pharmaceutical composition for gene therapy for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding a peptide-type compound described in items (1) to (16) above into cells in a living body and expressing a peptide with at least one modified amino acid, the peptide having the activity of increasing the intracellular calcium ion concentration.

(38) A method for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding a peptide-type compound described in items (1) to (16) above into cells in a living body enabling a peptide having an amino acid sequence encoded by said DNA to be produced as a peptide having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence, whereby a peptide having the activity of inducing growth hormone is expressed;

(39) A pharmaceutical composition for gene therapy for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding a peptide-type compound described in items (1) to (16) above into cells in a living body and expressing a peptide with at least one modified amino acid, the peptide having the activity of increasing the intracellular calcium ion concentration;

(40) A method for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding a peptide-type compound described in items (1) to (16) above into cells in a living body enabling a peptide having an amino acid sequence encoded by said DNA to be produced as a peptide having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence, whereby a peptide having the activity of inducing growth hormone is expressed.

**[0048]** Specifically, the present invention also relates to:

(1) A peptide-type compound having the activity of increasing the intracellular calcium ion concentration, wherein at least one amino acid is replaced by a modified amino acid and/or a non-amino acid compound, or a pharmaceutically acceptable salt thereof;

(2) The peptide-type compound according to item (1) above, which comprises an amino acid sequence set forth in SEQ ID NO: 1, or a pharmaceutically acceptable salt thereof;

(3) The peptide-type compound according to item (1) above, which comprises an amino acid sequence set forth in SEQ ID NO: 2 or an amino acid sequence wherein in SEQ ID NO: 2, at least one amino acid is deleted, replaced and/or added in a part outside a sequence of amino acids 1 to 7 from the amino-terminal thereof, or a pharmaceutically acceptable salt thereof;

(4) An analogue or derivative of the peptide described in item (1) above, which comprises an amino acid sequence set forth in SEQ ID NO: 3 or an amino acid sequence wherein in SEQ ID NO: 3, at least one amino acid is deleted, replaced and/or added in a part outside a sequence of amino acids 1 to 7 from the amino-terminal thereof, or a pharmaceutically acceptable salt thereof;

(5) A precursor peptide-type compound of the peptide compound described in (3) above, which comprises an amino acid sequence set forth in SEQ ID NO: 4 or an amino acid sequence wherein in SEQ ID NO: 4, at least one amino acid is deleted, replaced and/or added in a part outside a sequence of amino acids 1 to 28 from the amino-terminal thereof;

(6) A precursor peptide-type compound of the peptide compound described in item (4) above, which comprises an amino acid sequence set forth in SEQ ID NO: 5 or an amino acid sequence wherein in SEQ ID NO: 5, at least one amino acid is deleted, replaced and/or added in a part outside a sequence of amino acids 1 to 28 from the amino-terminal thereof;

(7) A peptide-type compound having the activity of increasing the intracellular calcium ion concentration and the activity of inducing secretion of growth hormone, or a pharmaceutically acceptable salt thereof;

(8) The peptide-type compound according to item (7) above, which has the activity of increasing the intracellular calcium ion concentration and the activity of inducing secretion of growth hormone and has at least one amino acid replaced by a non-amino acid compound, or a pharmaceutically acceptable salt thereof;

(9) The peptide-type compound according to items (7) to (8) above, which comprises an amino acid sequence set forth in SEQ ID NO: 1, or a derivative thereof or a pharmaceutically acceptable salt thereof;

(10) The peptide compound described in items (7) to (8) above, which comprises an amino acid sequence set forth in SEQ ID NO: 2 or an amino acid sequence wherein in SEQ ID NO: 2, at least one amino acid is deleted, replaced and/or added in a part outside a sequence of amino acids 1 to 7 from the amino-terminal thereof, or a derivative thereof or a pharmaceutically acceptable salt thereof;

(11) The peptide compound described in items (7) to (8) above, which comprises an amino acid sequence set forth in SEQ ID NO: 3 or an amino acid sequence wherein in SEQ ID NO: 3, at least one amino acid is deleted, replaced and/or added in a part outside a sequence of amino acids 1 to 7 from the amino-terminal thereof, or a derivative thereof or a pharmaceutically acceptable salt thereof;

- (12) A precursor peptide-type compound of the peptide-type compound described in item (10) above, which comprises an amino acid sequence set forth in SEQ ID NO: 4 or an amino acid sequence wherein in SEQ ID NO: 4, at least one amino acid is deleted, replaced and/or added in a part outside a sequence of amino acids 1 to 28 from the amino-terminal thereof;
- 5 (13) A precursor peptide-type compound of the peptide-type compound described in item (11) above, which comprises an amino acid sequence set forth in SEQ ID NO: 5 or an amino acid sequence wherein in SEQ ID NO: 5, at least one amino acid is deleted, replaced and/or added in a part outside a sequence of amino acids 1 to 28 from the amino-terminal thereof;
- (14) The peptide-type compound according to items (1) to (6) above, wherein the modified amino acid is an amino acid at the 3rd position from the amino-terminal thereof, or a pharmaceutically acceptable salt thereof;
- 10 (15) The peptide-type compound according to item (14), wherein the amino acid in the modified amino acid is serine or cysteine, or a pharmaceutically acceptable salt thereof;
- (16) The peptide-type compound according to items (1) to (6) above, wherein the modification in the modified amino acid indicates the modification at the  $\alpha$  carbon of said amino acid by (a) a saturated or unsaturated alkyl chain containing one or more carbon atoms which binds in a mode of linkage selected from the group consisting of ester, ether, thioester, thioether, amide or carbamide via or not via an alkyl chain containing one or more carbon atoms, or (b) H or a saturated or unsaturated alkyl chain containing one or more carbon atoms, or a pharmaceutically acceptable salt thereof;
- 15 (17) The peptide-type compound according to item (1) above, which comprises a modified amino acid modified with an ester linkage, or a pharmaceutically acceptable salt thereof;
- 20 (18) The peptide-type compound according to item (17) above, which comprises an amino acid to which a fatty acid is bound, or a pharmaceutically acceptable salt thereof;
- (19) The peptide-type compound according to item (18) above, which comprises an amino acid to which a fatty acid containing 2 to 35 carbon atoms is bound, or a pharmaceutically acceptable salt thereof;
- 25 (20) The peptide-type compound according to item (18) above, wherein the bound fatty acid is caprylic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof;
- (21) The peptide-type compound according to item (18) above, wherein the bound fatty acid is capric acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof;
- (22) The peptide-type compound according to item (18) above, wherein the bound fatty acid is lauric acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof;
- 30 (23) The peptide-type compound according to items (1) to (22) above, wherein the amino-terminal is modified with a saturated or unsaturated alkyl or acyl group containing one or more carbon atoms, and/or the carboxyl-terminal is OZ or NR<sub>2</sub>R<sub>3</sub> wherein Z is a pharmaceutically acceptable cation or a lower branched or linear alkyl group, and R<sub>2</sub> and R<sub>3</sub> are the same or different and represent H or a lower branched or linear alkyl group;
- 35 (24) A pharmaceutical composition for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises a peptide-type compound described in items (1) to (6) above or a pharmaceutically acceptable salt thereof as an active ingredient;
- (25) A pharmaceutical composition for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises an agent for treating diseases not attributable to a defect or decrease in growth hormone and a peptide-type compound described in items (1) to (6) above or a pharmaceutically acceptable salt thereof;
- 40 (26) A pharmaceutical composition according to items (24) to (25), which is applied to animals other than human beings;
- (27) A method for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises administering a pharmaceutical composition comprising a peptide-type compound described in items (1) to (6) above or a pharmaceutically acceptable salt thereof as an active ingredient;
- 45 (28) A method for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises administering an agent for treating diseases not attributable to a defect or decrease in growth hormone and a peptide-type compound described in items (1) to (6) above or a pharmaceutically acceptable salt thereof;
- (29) The treatment method according to items (27) to (28), which is applied to animals other than human beings;
- 50 (30) A DNA for a peptide-type compound described in items (1) to (6), which comprises a DNA sequence coding a peptide having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence;
- (31) The cDNA according to item (30) above, which comprises a DNA sequence set forth in SEQ ID NO: 6 (including NCR);
- 55 (32) The cDNA according to item (30) above, which comprises a DNA sequence of bases 31 to 381 in a DNA sequence set forth in SEQ ID NO: 6 (not including NCR);
- (33) The cDNA according to item (30) above, which comprises a DNA sequence set forth in SEQ ID NO: 7 (including NCR);

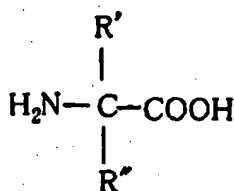


- (34) The cDNA according to item (30) above, which comprises a DNA sequence of bases 34 to 385 in a DNA sequence set forth in SEQ ID NO: 7 (not including NCR);
- (35) A vector comprising a DNA described in items (30) to (34) above;
- (36) Cells comprising the vector described in item (35) above;
- 5 (37) Cells comprising a vector containing a DNA described in items (30) to (34) above, wherein a peptide-type compound having an amino acid sequence encoded by said DNA can be produced as a peptide-type compound having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence;
- (38) An antibody against a peptide-type compound described in items (1) to (23) above;
- (39) A method for assaying a peptide-type compound described in items (1) to (23) above, which comprises using the antibody described in item (38) above to detect the peptide-type compound;
- 10 (40) A kit for detecting a peptide-type compound described in items (1) to (23) above, which comprises using the antibody described in item (38) above to detect the peptide-type compound;
- (41) A method for producing a peptide-type compound described in items (1) to (6) above by genetic recombination technology, which comprises transforming a vector containing a DNA described in item (30) above into host cells capable of modifying a side chain of at least one amino acid in said peptide, then culturing the resulting transformed cells and recovering the desired peptide-type compound from the culture;
- 15 (42) A method for producing a peptide-type compound described in items (1) to (6) above by genetic recombination technology, which comprises transforming a vector containing a DNA described in item (30) above into host cells, then culturing the resulting transformed cells and recovering the desired peptide-type compound from the culture, followed by chemically modifying an arbitrary amino acid thereof;
- (43) A method for producing a peptide-type compound described in items (18) to (22) above by genetic recombination technology, which comprises using cells enabling the peptide-type compound to be produced as a peptide having a fatty acid bound to a serine residue in the amino acid sequence set forth in SEQ ID NO: 1;
- (44) A method for producing a peptide-type compound having the activity of increasing the intracellular calcium ion concentration and the activity of secreting growth hormone, which comprises transforming a vector containing a DNA coding a peptide-type compound described in items (7) to (13) above into host cells, culturing the resulting transformed cells and recovering the desired compound from the culture;
- 25 (45) A pharmaceutical composition for gene therapy for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (6) above into cells in a living body and expressing a peptide with at least one modified amino acid, the peptide having the activity of increasing the intracellular calcium ion concentration;
- 30 (46) A method for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (6) above into cells in a living body enabling a peptide having an amino acid sequence encoded by said DNA to be produced as a peptide having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence, whereby a peptide having the activity of inducing growth hormone is expressed;
- 35 (47) A pharmaceutical composition for gene therapy for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (6) above into cells in a living body and expressing a peptide with at least one modified amino acid having the activity of increasing the intracellular calcium ion concentration;
- (48) A method for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding an amino acid sequence of a peptide-type compound described in items (1) to (6) above into cells in a living body enabling a peptide having an amino acid sequence encoded by said DNA to be produced as a peptide having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence, whereby a peptide having the activity of inducing growth hormone is expressed.
- 45

[0049] In the present invention, the amino acid encompasses every amino acid such as L-amino acid, D-amino acid,  $\alpha$ -amino acid,  $\beta$ -amino acid,  $\gamma$ -amino acid, natural amino acid and synthetic amino acid or the like.

50 [0050] In the present invention, the modified amino acid refers to an amino acid wherein an arbitrary group thereof is chemically modified. In particular, a modified amino acid chemically modified at the  $\alpha$ -carbon atom in an  $\alpha$ -amino acid is preferable. That is, when the  $\alpha$ -amino acid is represented by formula (1):

55



10 R' and R'' in the chemically modified amino acid may be H or an arbitrary group; in short, the modified amino acid may be any chemically modified natural amino acid. Either R' or R'' may also be H.

[0051] An amino acid wherein as the substituent group represented by R' and R'', a substituent group present in the natural amino acid is replaced by a substituent group not present in the natural amino acid or in its corresponding D-amino acid is referred to as the modified amino acid.

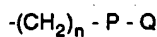
15 [0052] When the naturally occurring amino acid contains e.g. -OH, -SH, -NH or -NH<sub>2</sub> as a substituent group in a side chain thereof, a group formed by acylating such a substituent group is mentioned as a preferable example of the substituent group mentioned above.

[0053] The acyl group therefor includes e.g. groups formed by removing a hydroxyl group from an organic carboxylic acid, organic sulfonic acid and organic phosphoric acid.

20 [0054] The organic carboxylic acid includes e.g. fatty acids, and the number of carbon atoms thereof is preferably 2 to 35, more preferably 6 to 18 and most preferably 8 to 16. Such fatty acids include e.g. octanoic acid (preferably caprylic acid), decanoic acid (preferably capric acid), and dodecanoic acid (preferably lauric acid), as well as monoene or polyene fatty acids thereof.

[0055] In the organic sulfonic acid or organic phosphoric acid, the number of carbon atoms thereof is preferably 2 to 35.

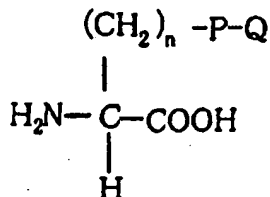
25 [0056] Further, the modified amino acid may be an amino acid wherein the group represented by R' and/or R'' is replaced, for example, by:



30 (wherein n is an integer of 0 to 10, P is -CO-O-, -O-CO-, -O-, -CO-S-, -CS-S-, -S-CO-, -S-, -CO-NH-, -NH-CO- or -CO-NH-CO-, Q is H or C<sub>1-35</sub>, preferably C<sub>1-20</sub>, alkyl). Further, P may be -CO-.

35 [0057] In addition, P may be -S-S- or -NH-CS-. In every -NH- described above, H may be replaced by a C<sub>1-35</sub> saturated or unsaturated alkyl group, a C<sub>6-20</sub> aryl group, or a C<sub>7-13</sub> aralkyl group.

[0058] In the case where the α-amino acid is represented by the formula (1) above, the modified amino acid wherein R' or R'' is replaced by the above group -(CH<sub>2</sub>)<sub>n</sub>-P-Q is a preferable embodiment. In particular, said modified amino acid is preferably modified serine wherein a substituent group represented by the above formula -(CH<sub>2</sub>)<sub>n</sub>-P-Q is bound to the α-carbon of serine, as shown in the formula:



40 wherein n, P and Q have the same meanings as defined above.

45 [0059] The mode of linkage selected from the group consisting of ester, ether, thioester, thioether, amide and carbamide via or not via an alkyl group containing one or more carbon atoms is described in more detail.

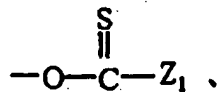
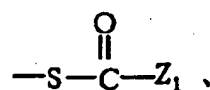
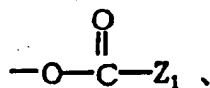
50 [0060] For example, if the amino acid is serine, threonine, tyrosine or oxyproline, the amino acid has a hydroxyl group in the side chain. If the amino acid is cysteine, the amino acid has a mercapto group in the side chain. If the amino acid is lysine, arginine, histidine, tryptophan, proline or oxyproline, it has an amino group or imino group in the

side chain.

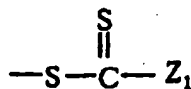
[0061] The hydroxyl group, mercapto group, amino group and imino group described above may have been chemically modified. That is, the hydroxyl group or mercapto group may be etherized, esterified, thioetherified or thioesterified. The imino group may have been iminoetherified, iminothioetherified or alkylated. The amino group may have been amidated, thioamidated or carbamidated.

[0062] Further, the mercapto group may have been disulfidated, the imino group may have been amidated or thioamidated, and the amino group may have been alkylated or thiocarbamidated.

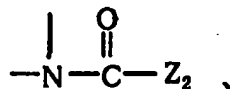
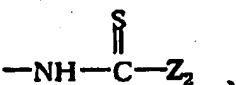
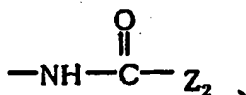
[0063] The thus chemically modified hydroxyl group or mercapto group can be represented for example by:



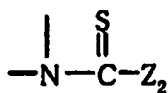
or



[0064] The amidated or thioamidated amino group or imino group can be represented by:



or



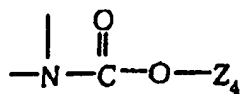
[0065] The etherified hydroxyl group or mercapto group can be represented by:



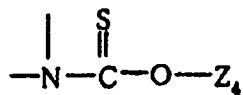
or



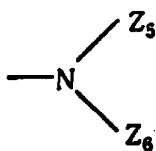
[0066] The iminoetherified or iminothioetherified imino group can be represented by:



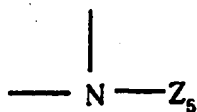
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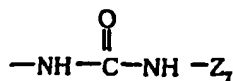
[0067] The alkylated amino group can be represented by:



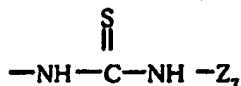
[0068] The alkylated imino group can be represented by:



[0069] The carbamidated or thiocarbamidated imino group can be represented by:



or



[0070] The disulfidated mercapto group can be represented by: -S-S-Z<sub>8</sub>

[0071] In the formulae above, Z<sub>1</sub>, Z<sub>2</sub>, Z<sub>3</sub>, Z<sub>4</sub>, Z<sub>5</sub>, Z<sub>6</sub>, Z<sub>7</sub> and Z<sub>8</sub> may be any substituent groups for chemical modification insofar as they are not against the spirit of the present invention, but because substituent groups used conventionally in a pharmaceutical field or for chemical modification of peptides are well known in patent literatures or scientific literatures, such known substituent groups for modification can be used and according to such known methods, chemical modification can be performed in the present invention.

[0072] In the above formulae, Z<sub>1</sub> may be a hydrogen atom or a linear-chain, branched or cyclic alkyl group, and such alkyl group may be saturated or unsaturated. The number of carbon atoms thereof is usually C<sub>1-50</sub>, preferably C<sub>6-20</sub>.

[0073] Z<sub>2</sub>, Z<sub>3</sub>, Z<sub>4</sub>, Z<sub>5</sub>, Z<sub>6</sub>, Z<sub>7</sub> or Z<sub>8</sub> may be a hydrogen atom or a straight-chain, branched or cyclic alkyl group, and such alkyl group may be saturated or unsaturated. The number of carbon atoms thereof is usually C<sub>1-10</sub>, preferably C<sub>1-6</sub>.

[0074] The alkyl groups represented by Z<sub>1</sub>, Z<sub>2</sub>, Z<sub>3</sub>, Z<sub>4</sub>, Z<sub>5</sub>, Z<sub>6</sub>, Z<sub>7</sub> or Z<sub>8</sub> may be substituted with substituent groups such as hydroxyl group, amino group, halogen, nitro group and C<sub>1-3</sub> alkoxy group, which are used conventionally for chemical modification of peptides.

[0075] In the above, if Z<sub>1</sub>-CO- is a residue of the fatty acid Z<sub>1</sub>-COOH, this is one example of the amino acid to which the fatty acid was bound. The fatty acid in this case includes e.g. saturated fatty acid such as caprylic acid, capric acid, lauric acid, butyric acid, caproic acid, undecylic acid, palmitic acid, decanoic acid, nonadecanoic acid, behenic acid, montanic acid and lacceric acid and unsaturated fatty acid such as acrylic acid, oleic acid, linolic acid, linolenic acid and aatearolic acid. The unsaturated fatty acid may be a monoene or a polyene.

[0076] Further, the modified amino acid may also be an α-amino acid formed by replacing a group (excluding a carboxyl group and an amino group constituting a peptide linkage) binding to the α-carbon atom of the α-amino acid by a hydrogen atom or a saturated or unsaturated alkyl group.

[0077] In the present invention, the modified amino acid may also be an amino acid formed by introducing a C<sub>1-6</sub> saturated or unsaturated alkyl group onto the amino group of the amino acid.

[0078] The non-natural amino acid in the present invention is the one having amino group and carboxyl group in both terminals of the molecule, and includes e.g. NH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>CH(CH<sub>2</sub>OH)-COOH, NH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-COOH, NH<sub>2</sub>-C(CH<sub>3</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>-COOH, and NH<sub>2</sub>-CH(CH<sub>3</sub>)-(CH<sub>2</sub>)<sub>2</sub>-CH(CH<sub>3</sub>)-COOH. The length of their molecular chain corresponds to the length of a dipeptide, but the non-natural amino acid in the present invention also includes those having the length of a peptide.

[0079] Further, the non-amino acid compound in the present invention includes e.g. NH<sub>2</sub>-CH(CH<sub>2</sub>OH)-CH<sub>3</sub>, CH<sub>3</sub>-CH(R)-COOH, CH<sub>3</sub>-CH(R)-CH<sub>3</sub> wherein the length of their molecule corresponds to the length of a peptide, or NH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>CH(CH<sub>2</sub>OH)-CH<sub>3</sub> and NH<sub>2</sub>-(CH<sub>2</sub>)<sub>3</sub>CH(R)-CH<sub>3</sub> wherein the length of their molecule corresponds to the length of a dipeptide.

[0080] Here, R represents a substituent group on a side chain of the natural amino acid or on the α-carbon in the aforementioned modified amino acid.

## Brief Description of Drawings

[0081]

Fig. 1 shows purification of ghrelin from rat stomach extract, and the change in fluorescence intensity by an increase in the intracellular calcium ion concentration in CHO-GHSR62 cells is shown by the black bar. Fig. 1a shows a profile in Sephadex G-50 (fine) gel filtration of an SP-III fraction prepared from 40 g rat stomach, to indicate that the molecular weight of the active fractions is about 3,000 Dalton. Fig. 1b is a graph shows a profile in secondary CM ion-exchange HPLC, and the active fractions eluted at retention times of 55 to 56 minutes were further purified

by reversed-phase HPLC.

Fig. 2 shows that the modification of ghrelin with n-octanoyl was identified. Fig. 2a shows the result of analysis of 2 µg each of natural ghrelin (upper) and synthetic ghrelin and synthetic de-acylated ghrelin (lower) by reversed-phase HPLC. Fig. 2b is a graph showing changes in the intracellular calcium ion concentration in CHO-GHSR62 cells by natural ghrelin (solid line), synthetic ghrelin (small broken line) and synthetic de-acylated ghrelin (large solid line).

Fig. 3 is a graph showing specific interaction of ghrelin with CHO-GHSR62 cells, and ghrelin was added at the arrowed point. Fig. 3a is a graph showing changes in the intracellular calcium ion concentration in CHO-GHSR62 cells by ghrelin, GHRP-6 and GRF (GHRH), respectively. Fig. 3b is a graph showing changes in the intracellular calcium ion concentration in CHO-GHSR62 cells by ghrelin in the presence (○) or absence (●) of [D-Lys-3]-GRP-6, and a change in the intracellular calcium ion concentration by GRF (GHRH) (black triangle) is also shown.

Fig. 4 shows the amino acid sequences of rat- and human-derived ghrelin precursors, as well as the analysis result of expression of these precursors in various tissues. Fig. 4a shows comparison between the amino acid sequences of rat- and human-derived ghrelin precursors, where the same amino acid is shaded, a signal peptide is indicated by the broken line, a cleavage site of the signal peptide is indicated by the shaded triangle, a cleavage site at the side of carboxyl-terminal is indicated by the triangle, a matured ghrelin moiety is boxed, and a modification with n-octanoic acid is indicated by \*. Fig. 4b shows the analysis result of expression of ghrelin in a wide variety of rat tissues by Northern blotting.

Fig. 5 is a graph showing the effect of ghrelin *in vitro* and *in vivo* on secretion of pituitary hormones. Fig. 5a is a graph showing a change in fluorescence intensity by a change in the intracellular calcium ion concentration in rat pituitary cultured cells at an initial stage, where the change upon addition of ghrelin is indicated by the solid line and the change upon addition of de-acylated ghrelin by the broken line. Fig. 5b is a graph showing the secretion of pituitary hormones, where the black bar and white bar show the concentrations of pituitary hormone levels in the presence and absence of ghrelin, respectively. Fig. 5c is a graph showing a time course of pituitary hormone concentration in plasma after ghrelin was injected intravenously to male rats. In Fig. 5b and 5c, GH is growth hormone, ACTH is adrenocorticotropin, FSH is follicle-stimulating hormone, LH is luteinizing hormone, PRL is prolactin, and TSH is thyroid-stimulating hormone.

Fig. 6 shows promotion of appetite upon administration of ghrelin into ventricle, where the amount of feed (mean±standard error) for 2 hours after administration of ghrelin is shown. Fig. 6a shows that the error range for the effect of ghrelin is less than 0.0001.

Fig. 7 shows the effect of a drug administered to rat under urethane anesthesia on secretion of gastric acid, where A and B show the results of administration of rat ghrelin (rGhrelin) and histamine, respectively. Each symbol indicates an average value from 4 rats, and the standard error is shown by an error bar. As the control, physiological saline was administered. At the arrowed point, the drug was administered.

Fig. 8 is a graph showing the action of rat ghrelin on stomach motility in rat under urethane anesthesia. Fig. 8A shows typical waves of stomach motility upon administration of physiological saline and rat ghrelin (rGhrelin), and Fig. 8B is a graph showing an average value from 4 rats along with the standard error. At the arrowed point, the drug was administered.

Fig. 9 is a graph showing a standard curve in radioimmunoassays and cross-reactivity with antibody. Fig. 9a is a graph showing the binding inhibition, by various ghrelins, of <sup>125</sup>I-labeled rat ghrelin to an antibody against a amino-terminal ghrelin fragment, and Fig. 9b is a graph showing the binding inhibition, by various ghrelins, of <sup>125</sup>I-labeled rat ghrelin to an antibody against a carboxyl-terminal ghrelin fragment. The amount of various ghrelins/reaction tube is shown on the abscissa, while the ratio (%) of the amount (B) of rat ghrelin bound in the presence of various ghrelins to the amount thereof (B<sub>0</sub>) in the absence of various ghrelins is shown on the ordinate. Symbols in the graphs are as follows: Rat ghrelin (○); human ghrelin (●); rat ghrelin-27 (□); [Ser3(decanoyl)]-rat ghrelin (◇); [Ser3(hexanoyl)]-rat ghrelin (Δ); and de-fatty acid rat ghrelin (▼).

#### Best Mode for Carrying Out the Invention

**[0082]** For a peptide serving as an endogenous ligand for GHS receptor (GHS-R), the distribution of the endogenous ligand in organs or tissues can be known by adding an extract from various organs or tissues to cells expressing GHS-R and measuring the intracellular calcium ion concentration.

**[0083]** The cells expressing GHS-R include strains derived from the hypothalamus and pituitary gland known to express GHS-R constantly and their tissues, but the cells are preferably those transformed cells having GHS-R gene introduced into suitable cells such as CHO cells, and expressing the gene.

**[0084]** The strong Ca-releasing activity of the endogenous GHS peptide of the present invention was found not in the hypothalamus and pituitary gland expressing the peptide, but in an extract from stomach as an organ in the digestive organ system. It is therefore necessary to examine not only tissues and organs expressing said receptor but also a

wide variety of other tissues and organs in order to find the desired endogenous ligand for the orphan receptor.

[0085] The intracellular calcium ion concentration can be measured by any method known in the art, preferably by means of FLIPR (Fluorometric Imaging Plate Reader, Molecular Devices Co., Ltd.) utilizing the change in the fluorescent intensity of Fluo-4 AM (Molecular Probe Co., Ltd.) caused by a change in the concentration of calcium ions.

5 [0086] To obtain the desired endogenous GHS peptide from tissues and organs confirmed to exhibit the Ca-releasing activity, any purification method known in the art can be used.

[0087] As the method of purifying the peptide, it is effective to use singly or in combination of gel filtration, ion exchange and reversed-phase chromatographic techniques after a wide variety of fractionation methods or use them separately, but it is possible to use not only such chromatographic techniques but also any means effective for purification of the peptide.

10 [0088] For isolation and purification of the peptide from the tissues and organs, inactivation of proteases in the tissues and organs by heat treatment thereof in boiling water is desired to prevent degradation of the desired peptide by the action of the proteases. Heat treatment and removal of the tissues and organs under cooling on ice are also effective for extraction and purification of the desired peptide.

15 [0089] To confirm that the purified peptide having the Ca-releasing activity has a GH secretion-inducing activity *in vitro* and *in vivo*, a known method can be utilized.

[0090] For example, GH secreted into a medium of pituitary gland cell culture confirmed to secrete GH and express GHS-R can be measured *in vitro* in radioimmunoassays by adding anti-GH antibody to the cells. By using an antibody against other hormone in place of the anti-GH antibody in radioimmunoassays, the amount of said hormone secreted

20 can also be measured.

[0091] Further, the GH secretion-inducing activity *in vivo* can be confirmed by injecting the peptide having the Ca-releasing activity into a peripheral vein of an animal and then measuring the concentration of GH in serum.

[0092] For analyzing the structure of the purified peptide, a known method can be used.

25 [0093] For determining the amino acid sequence of the peptide, there is a method wherein amino acid residues are released sequentially from the carboxyl-terminal by Edman degradation followed by identification of the released amino acids by high performance liquid chromatography (HPLC), as well as an automated version thereof by an amino acid sequencer.

[0094] There is also a method for determining the amino acid sequence thereof by measuring the molecular weights of ionized fragments thereof by GC-MASS.

30 [0095] For the peptide containing modified amino acids in one aspect of the present invention, the modified amino acid is identified as "unknown amino acid" upon determination of the amino acid sequence.

[0096] In this case, the modified peptide is decomposed into amino acid units from which the modified amino acid is separated and purified, and the structure of the modified amino acid is determined in a usual manner for determining the structure of the compound, whereby the entire structure of the peptide can be known. Alternatively, there is a method wherein the peptide is obtained from a cDNA coding the modified peptide, then a peptide having the amino acid sequence of the resulting peptide is chemically synthesized, and the molecular weight and physical properties of the synthetic unmodified peptide are compared with those of the modified peptide in order to estimate the structure of the modified group.

35 [0097] A partial amino acid sequence (core sequence) which in the peptide thus structurally determined, is essential for the Ca-releasing activity is revealed by measuring the Ca-releasing activity of each peptide fragment formed by cleaving said peptide with a protease.

[0098] The used protease shall be a protease highly specific to the amino acid sequence of the peptide to be cleaved, but a low specific protease can also be used under conditions for partial digestion to prepare various peptide fragments from said peptide.

40 [0099] By measuring the Ca-releasing activity of each peptide fragment thus prepared, a core sequence essential for the Ca-releasing activity can be known.

[0100] In the endogenous GH secretion-inducing peptide, 3rd serine from the amino-terminal has been acylated with a fatty acid, and it is also possible to chemically be synthesized a peptide fragment having a part of the amino acid sequence of the endogenous GH secretion-inducing peptide, as well as a fatty acid-modified peptide comprising a fatty acid bound via an ester linkage to the serine side chain in said peptide fragment.

50 [0101] Using said peptide fragment, the endogenous GH secretion-inducing peptide can be analyzed in detail. Simultaneously, the type of fatty acid necessary for the Ca-releasing activity can be determined by comparing the peptide fragments modified with various fatty acids.

55 [0102] For example, in the endogenous GH secretion-inducing peptide derived from some species of *Xenopus Laevis*, an amino-acid residue at the 3rd position from the amino-terminal is not serine but threonine, and such threonine has been acylated with a fatty acid, and this peptide-type compound can also be synthesized and said compound can be analyzed in detail.

[0103] By comparing the amino acid sequences of those peptides having a GH secretion-inducing activity in verte-

brates, a region preserved widely in vertebrates can be found, and from the amino acid sequence of said region, a core sequence essential for the GH secretion-inducing activity can be found.

[0104] A DNA having a nucleotide sequence deduced from the amino acid sequence of the endogenous GH secretion-inducing peptide is chemically synthesized, and this DNA is used as a probe for screening a cDNA library prepared from mRNA in cells expressing said peptide, whereby a cDNA coding said peptide can be obtained.

[0105] However, a codon corresponding to an amino acid is degenerated thus increasing the number of nucleotide sequences deduced from the amino acid sequence of the peptide, so that the screening by using a certain synthetic DNA consisting of various types of such nucleotide sequences as a probe can be difficult.

[0106] In this case, if a sequence in accordance with the amino acid sequence of said peptide is present in amino acid sequences deduced from the nucleotide sequence of an expressed sequence tag (EST) disclosed in a sequence data base, a DNA consisting of a part of the nucleotide sequence of the EST can be synthesized and used to screen the above cDNA library.

[0107] Further, a genomic DNA can be obtained in a usual manner from the cDNA.

[0108] From the nucleotide sequence of the cDNA thus obtained, the amino acid sequence of a precursor polypeptide of the endogenous GH secretion-inducing peptide is revealed.

[0109] By analyzing said amino acid sequence, a signal peptide, the endogenous GH secretion-inducing peptide, other peptide moieties and cleavage sites of these peptides are revealed, thus revealing the mechanism of formation of the endogenous GH secretion-inducing peptide.

[0110] Other aspects of the present invention, that is, a partial amino acid sequence of the endogenous GH secretion-inducing peptide, the amino acid sequence of a precursor polypeptide of said peptide, and the nucleotide sequence of a DNA coding said polypeptide are disclosed in International Appln. Disclosure WO 98/42840, but the peptide disclosed therein is a peptide consisting of 14 amino acids having a motilin-like activity, and there is no description therein of the activity of increasing Ca concentration and the activity of inducing GH secretion disclosed in the present invention.

[0111] The peptide-type compound of the present invention refers to a peptide having the activity of increasing the intracellular calcium ion concentration, which is represented by formula (2) below wherein at least one amino acid is replaced by a modified amino acid; a peptide analogue thereof wherein at least one amino acid is replaced by a non-amino acid; and a peptide derivative thereof wherein amino-terminal and/or carboxyl-terminal is modified.

[0112] In the present invention, the peptide, peptide analogue and peptide derivative described above are referred to collectively as the peptide-type compound.

[0113] In the peptide-type compound, a plurality of amino acids may be replaced by modified amino acids and/or non-amino acids. In the amino acid sequence set forth in SEQ ID No:2, it is preferable in the present invention that usually one or more amino acids of the 1st to 10th amino acids from the amino-terminal, preferably the 1st to 4th amino acids or the 1st to 5th amino acids from the amino-terminal are replaced by modified amino acids and/or non-amino acids. It is particularly preferable that the 1st to 5th amino acids are replaced by modified amino acids and/or non-amino acids.

[0114] In the amino acid sequence set forth in SEQ ID NO: 2, one or more amino acids outside of the 1st to 4th amino acids, preferably the 1st to 6th amino acids and more preferably the 1st to 10th amino acids from the amino-terminal may be added or deleted.

[0115] The peptide-type compound of the present invention is preferably a peptide compound which has the activity of increasing the intracellular calcium ion concentration and induces secretion of growth hormone *in vivo*, wherein at least one amino acid is replaced by a modified amino acid and/or a non-amino acid compound.

[0116] That is, the peptide-type compound of the present invention is a peptide-type compound having the activity of increasing the intracellular calcium ion concentration and/or the action of inducing secretion of growth hormone *in vivo*, wherein an amino acid in the peptide chain is replaced by a modified amino acid and/or a non-amino acid compound.

[0117] Examples of the compound include those compounds wherein in the peptide shown in SEQ ID NO: 1, 2 or 3, a hydroxyl group of the amino acid Ser 3 is acylated, those compounds wherein in the peptide shown in SEQ ID NO: 4 or 5, a hydroxyl group of the 25th amino acid Ser is acylated, or pharmaceutically acceptable salts thereof.

[0118] Other examples include those compounds wherein in the peptide shown in SEQ ID NO: 10, 11, 16 or 17, a hydroxyl group of the amino acid Ser 3 is acylated, or pharmaceutically acceptable salts thereof.

[0119] Still other examples include those compounds wherein in the peptide shown in SEQ ID NO: 22, 25, 26 or 27, a hydroxyl group of the amino acid Ser 3 is acylated, or pharmaceutically acceptable salts thereof.

[0120] Still other examples include those compounds wherein in the peptide shown in SEQ ID NO: 29, 30 or 31, a hydroxyl group of the amino acid Ser 3 is acylated, or pharmaceutically acceptable salts thereof.

[0121] Still other examples include those compounds wherein in the peptide shown in SEQ ID NO: 28, a hydroxyl group of the amino acid Thr 3 is acylated, or pharmaceutically acceptable salts thereof.

[0122] The acyl group introduced into a hydroxyl group by acylation in the present invention is a group formed by removing a hydroxyl group from e.g. an organic carboxylic acid, an organic sulfonic acid or an organic phosphoric acid.



[0123] The organic carboxylic acid includes e.g. fatty acids, and the number of carbon atoms thereof is preferably about 2 to 35, more preferably about 6 to 18, and most preferably about 8 to 16. Such fatty acids include e.g. octanoic acid (preferably caprylic acid), decanoic acid (preferably capric acid), and dodecanoic acid (preferably lauric acid [sic: lauric acid]), as well as their monoene or polyene fatty acids thereof.

[0124] In the organic sulfonic acid or organic phosphoric acid, the number of carbon atoms thereof is preferably about 2 to 35.

[0125] Any peptide-type compounds or pharmaceutically acceptable salts thereof, including the amino acid sequence set forth in SEQ ID NO: 1 wherein a hydroxyl group of 3rd Ser is acylated, are also preferable embodiments of the present invention.

[0126] That is, in the second aspect of the present invention, any peptide-type compounds or pharmaceutically acceptable salts thereof, including fatty acid-modified peptides wherein a hydroxyl group of 3rd Ser is acylated in the amino acid sequence set forth in SEQ ID NO: 8, preferably the amino acid sequence set forth in SEQ ID NO: 1 and more preferably the amino acid sequence set forth in SEQ ID NO: 9, are also preferable embodiments of the present invention.

[0127] Further, any peptide compounds or pharmaceutically acceptable salts thereof, including fatty acid-modified peptides wherein a hydroxyl group of Thr 3 is acylated in the amino acid sequence set forth in SEQ ID NO: 8, preferably the amino acid sequence set forth in SEQ ID NO: 1 and more preferably an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO: 9, the amino acid residue serine at the 3rd position from the amino-terminal is converted into threonine, are also preferable embodiments of the present invention.

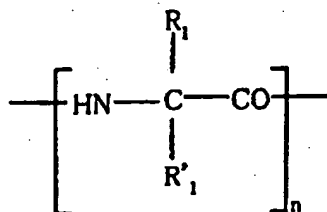
[0128] Further, a preferable embodiment of the present invention is a compound or pharmaceutically acceptable salts represented by formula (2):



(2)

wherein X is a moiety corresponding to a hydrogen atom in an amino group of the amino-terminal amino acid and represents H or a saturated or unsaturated alkyl or acyl group containing one or more carbon atoms; Y is a moiety corresponding to a hydroxyl group in an  $\alpha$ -carboxyl group of the carboxyl-terminal amino acid and represents OH, OZ or NR<sub>6</sub>R<sub>7</sub> whereupon Z is a pharmaceutically acceptable cation or a lower branched or linear alkyl group; and R<sub>6</sub> and R<sub>7</sub> may be the same or different and represent H or a lower branched or linear alkyl group.

[0129] Here, AA1 represents:

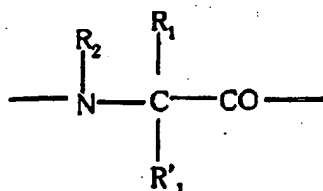


wherein n is 1 or 2, R<sub>1</sub> and R<sub>1</sub>' may be the same or different and represent hydrogen or a substituent group, provided that when n is 2, the two substituent groups R<sub>1</sub> may be the same or different; this also applies to R<sub>1</sub>'.

[0130] Examples of the substituent group include (1) a saturated or unsaturated alkyl chain containing one or more carbon atoms which binds in a mode of linkage selected from the group consisting of ester, ether, thioester, thioether, amide and carbamide via or not via an alkyl chain containing one or more carbon atoms, (2) H or a saturated or unsaturated alkyl chain containing one or more carbon atoms, and (3) a side chain of natural amino acid.

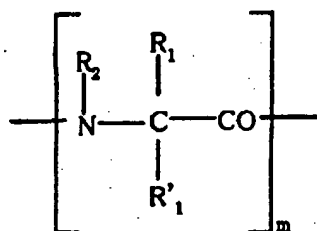
[0131] Further, the substituent group may be a saturated or unsaturated alkyl chain containing one or more carbon atoms, which is bound via a disulfide or thiocarbamide linkage via or not via an alkyl chain containing one or more carbon atoms.

[0132] AA2 represents:



wherein  $R_1$  and  $R'_1$  have the same meanings as defined above, and  $R_2$  represents H or a saturated or unsaturated alkyl group containing 1 to 6 carbon atoms, or AA2 represents  $-\text{CH}_2-\text{CH}(R_1)-\text{CH}_2-$  or  $-\text{CH}_2-\text{CH}(R_1)-\text{CO}-$  whereupon  $R_1$  has the same meaning as defined above.

[0133] AA3 represents:



wherein  $m$  is an integer of 1 or more, and  $R_1$ ,  $R'_1$  and  $R_2$  have the same meanings as defined above, provided that when  $m$  is an integer of 2 or more, the two substituent groups  $R_1$  may be the same or different; this also applies to  $R'_1$  and  $R_2$ .

[0134] The saturated or unsaturated alkyl containing one or more carbon atoms, which is represented by X, is preferably  $\text{C}_{1-20}$  alkyl such as methyl, ethyl, n-propyl, i-propyl, n-butyl, s-butyl, t-butyl, n-heptyl, n-hexyl, n-decyl, vinyl, propargyl or hexenyl.

[0135] The acyl represented by X includes  $\text{C}_{1-10}$  carboxylic acid acyl such as formyl, acetyl, propionyl or benzoyl, or  $\text{C}_{7-13}$  sulfonic acid acyl such as benzenesulfonyl naphthalene sulfonyl or the like.

[0136] The group represented by  $R_1$  or  $R'_1$  is preferably a group represented by e.g. formula (2):



(wherein  $n$  is an integer of 0 to 10, P is  $-\text{CO}-\text{O}-$ ,  $-\text{O}-\text{CO}-$ ,  $-\text{O}-$ ,  $-\text{CO}-\text{S}-$ ,  $-\text{CS}-\text{S}-$ ,  $-\text{S}-\text{CO}-$ ,  $-\text{S}-$ ,  $-\text{CO}-\text{NH}-$ ,  $-\text{NH}-\text{CO}-$  or  $-\text{CO}-\text{NH}-\text{CO}-$ , Q is H or  $\text{C}_{1-20}$  alkyl represented by X described above.) Further, P may also be  $-\text{CO}-$ .

[0137] In addition, P may be  $-\text{S}-\text{S}-$  or  $-\text{NH}-\text{CS}-$ . In every  $-\text{NH}-$  described above, H may be replaced by a  $\text{C}_{1-35}$  saturated or unsaturated alkyl group, a  $\text{C}_{6-20}$  aryl group, or a  $\text{C}_{7-13}$  aralkyl group.

[0138] More preferably, P is:

$-\text{CO}-\text{O}-$ ,  $-\text{CO}-$ ,  $-\text{O}-$ ,  $-\text{S}-$ ,  $-\text{S}-\text{S}-$ ,  $-\text{CO}-\text{S}-$ ,  $-\text{CO}-\text{NH}-$ ,  $-\text{NH}-\text{CO}-$  or  $-\text{NH}-\text{CS}-$ .

[0139] The group represented by  $R_1$  or  $R'_1$  may be a group having Q bound to  $-(\text{CH}_2)_n$  directly not via P.

[0140] It is preferable that the lower alkyl group represented by Z, R6 or R7 is  $\text{C}_{1-6}$  alkyl such as methyl, ethyl, n-propyl, i-propyl, n-butyl, s-butyl, t-butyl, i-butyl, n-pentyl or n-hexyl.

[0141] Hereinafter, preferable embodiments of the peptide compounds according to the present invention are described.

(1) Preferable embodiments of AA1: (A) amino acids or peptides such as Ser, Gly-Ser or  $-\text{NH}-(\text{CH}_2)_3\text{CH}(\text{CH}_2\text{OH})\text{CO}-$  where a peptide linkage moiety between two amino acid residues is  $-(\text{CH}_2)_2-$  and (B) primary amines, for example  $-\text{NH}-(\text{CH}_2)_3\text{CH}(\text{CH}_2\text{OH})\text{CH}_2-$  where a peptide linkage moiety between two amino acids is  $-(\text{CH}_2)_2-$ ;  $-\text{NH}-(\text{CH}_2)_3\text{CH}(R_1)\text{CH}_2-$  where a peptide linkage moiety between two amino acids is  $-(\text{CH}_2)_2-$ , wherein  $R_1$  has the same meanings as defined above; and  $-\text{NH}-\text{CH}(\text{CH}_2\text{OH})\text{CH}_2-$ .

As (A) amino acids or peptides,  $\text{NH}_2-(\text{CH}_2)_4-\text{COOH}$ ,  $\text{NH}_2-\text{C}(\text{CH}_3)_2-(\text{CH}_2)_3-\text{COOH}$  and  $\text{NH}_2-\text{CH}(\text{CH}_3)-(\text{CH}_2)_2-\text{CH}(\text{CH}_3)-\text{COOH}$  can also be exemplified.

(2) Preferable embodiments of AA2 : (A) amino acids such as Ser, homoSer, Cys, homoCys, Asp, Glu, Lys, Ala, Val, Leu, homoLeu, ile, homolle, ornithine, aminoadipic acid, methionine, ethionine, butionine, and S-methyl cysteine, among which Ser is particularly preferable, and (B) structures other than amino acid residues; for example, there can be mentioned  $-\text{CH}_2-\text{CH}(\text{R}_1)-\text{CO}-$ ,  $-\text{CH}_2-\text{CH}(\text{R}_1)-\text{CH}_2-$ , etc. wherein  $\text{R}_1$  has the same meanings as defined above.

In particular, amino acids (a) with a hydrophobic side chain, such as leucine, valine, norleucine, homoleucine, homoisoleucine, naphthyl alanine or its analogues thereof, tryptophan, phenylalanine, cyclohexylalanine etc. or N-methylamino acids thereof are preferable. Further, amino acids (b) with a side chain having a functional group capable of modification with acyl group, alkyl group, alkenyl group or aralkyl group, such as serine, homoserine, threonine, cysteine, homocysteine, aspartic acid, glutamic acid, adipic acid, lysine, ornithine etc. and N-methylamino acids thereof are preferable.

An acyl group, alkyl group, alkenyl group or aralkyl group etc. are bound to side chains of the amino acids (b) via an ester, amide, disulfide, ether, thioether, thioester, carbamide or thiocarbamide linkage. Further, an alkyl or aralkyl group may be bound to the  $\alpha$ -carbon of the amino acid.

(3) Preferable embodiments of AA3: Amino acids or peptides such as Phe or a peptide having an amino acid sequence of from the 4th Phe to the 28th Arg from the amino-terminal in the amino acid sequence set forth in SEQ ID NO: 2 or 3, or peptides having an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO: 2 or 3, one amino acid is sequentially deleted starting from the carboxyl-terminal amino acid until the 5th Leu from the amino-terminal. For example, AA3 includes:

Phe Leu,

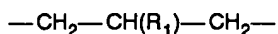
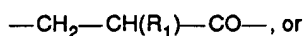
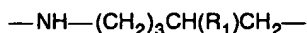
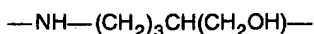
Phe Leu Ser,

Phe Leu Ser Pro,

Phe Leu Ser Pro Glu,

Phe Leu Ser Pro Glu His,  
 Phe Leu Ser Pro Glu His Gln,  
 5 Phe Leu Ser Pro Glu His Gln Arg,  
 Phe Leu Ser Pro Glu His Gln Lys Ala,  
 10 Phe Leu Ser Pro Glu His Gln Lys Ala Gln,  
 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln,  
 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg,  
 15 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys,  
 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu,  
 20 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser,  
 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys,  
 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys  
 25 Lys,  
 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys  
 Lys Pro,  
 30 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys  
 Lys Pro Pro,  
 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys  
 35 Lys Pro Pro Ala,  
 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys  
 40 Lys Pro Pro Ala Lys,  
 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys  
 Lys Pro Pro Ala Lys Leu,  
 45 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys  
 Lys Pro Pro Ala Lys Leu Gln,  
 50 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys  
 Lys Pro Pro Ala Lys Leu Gln Pro, and  
 Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys  
 55 Lys Pro Pro Ala Lys Leu Gln Pro Arg.

[0142] As a matter of course, the amino acids exemplified as AA3 may be L-amino acids or D-amino acids. Further, in the amino acid sequences exemplified as AA3, one to several amino acids (preferably up to about 1/3 of the amino acid sequence) may be replaced by non-natural amino acid units or non-amino acid units, for example:



wherein R<sub>1</sub> has the same meanings as defined above. When AA3 contains a plurality of groups represented by the above formulae and a plurality of groups represented by R<sub>1</sub>, these groups are the same or different.

[0143] Further, any amino acids exemplified as AA3 may have substituent groups represented by R<sub>1</sub> described above. When a plurality of R<sub>1</sub> groups are present in a group represented by AA3, these R<sub>1</sub> groups may be the same or different.

[0144] When amino acids constituting the peptide have hydroxyl group, mercapto group, imino group or amino group in their side chains, preferable examples of such side chains are shown below. In the following examples, R<sub>8</sub> is a saturated or unsaturated alkyl group containing one or more carbon atoms. Such alkyl chain may have the same meanings as defined for the above-described alkyl chain shown by X.

A) Side chain of Ser; -CH<sub>2</sub>-O-CO-R<sub>8</sub> or -CH<sub>2</sub>-O-R<sub>8</sub>,

B) Side chain of homoSer; -CH<sub>2</sub>-CH<sub>2</sub>-O-CO-R<sub>8</sub> or -CH<sub>2</sub>-CH<sub>2</sub>-O-R<sub>8</sub>,

C) Side chain of Cys; -CH<sub>2</sub>-S-CO-R<sub>8</sub> or -CH<sub>2</sub>-S-R<sub>8</sub>,

D) Side chain of homoCys; -CH<sub>2</sub>-CH<sub>2</sub>-S-CO-R<sub>8</sub> or -CH<sub>2</sub>-CH<sub>2</sub>-S-R<sub>8</sub>,

E) Side chain of Asp; -CH<sub>2</sub>-COO-R<sub>8</sub> or -CH<sub>2</sub>-CO-NH-R<sub>8</sub>,

F) Side chain of Glu; -CH<sub>2</sub>-CH<sub>2</sub>-COO-R<sub>8</sub> or -CH<sub>2</sub>-CH<sub>2</sub>-CO-NH-R<sub>8</sub>

G) Side chain of Lys; -(CH<sub>2</sub>)<sub>4</sub>-NH-CO-R<sub>8</sub>,

H) Side chain of amino adipic acid; -CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-COO-R<sub>8</sub> or -CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CO-NH-R<sub>8</sub>,

I) Side chain of ornithine; -(CH<sub>2</sub>)<sub>3</sub>-NH-CO-R<sub>8</sub>.

J) An alkyl side-chain in an amino acid such as Ala, Val, Leu, homoleucine, Ile, homoisoleucine, S-methyl cysteine, methionine, ethionine, or butionine may be a modified alkyl group shown in the formula (2) as described above.

[0145] Further, the present invention encompasses, as preferable embodiments, an agent for increasing the intracellular calcium ion concentration or an agent for inducing GH secretion, which comprises a partial peptide consisting of amino acids from the amino-terminal to 13th, 14th or 15th amino acids in the amino acid sequence of SEQ ID NO: 2 or 3. In this case, it is not always necessary that the respective amino acid units constituting the partial peptide are chemically modified.

[0146] Further, a preferable embodiment of the present invention is the following peptide-type compound.

[0147] A ghrelin derivative refers to a peptide-type compound wherein the chemical structure of natural ghrelin is partially modified, and short-chain ghrelin refers to a peptide consisting of less than 27 or 28 amino acids, which is derived from natural ghrelin of 27 to 28 amino acids by deleting some of the amino acids. Further, an amino acid residue at the n-position refers to an amino acid residue at the n-position from the amino-terminal.

[0148] The amino-terminal amino acid of ghrelin or its short-chain ghrelin derivative may be any amino acid (the amino-terminal amino acid of natural ghrelin is glycine) insofar as the α-amino group of said amino acid is not protected, or may be either D- or L-amino acid, but it is preferably alanine, valine, aminoisobutanoic acid or butanoic acid.

[0149] The 2nd residue may be any amino acid (e.g. serine in the natural ghrelin), preferably an amino acid having

a small side-chain, such as alanine, serine, histidine, norvaline or a non-amino acid compound.

[0150] The 1st and 2nd residues may be a  $\delta$ -amino acid corresponding to two amino acids, for example, 5-aminopentanoic acid, 5-amino-5-dimethylpentanoic acid, 2,5-diaminopentanoic acid etc. exemplified in the Examples.

[0151] The amino acid residues selected at the 3rd and 4th positions may be D- or L-amino acids, D- or L-N-methylamino acids, or a combination of these amino acids. In particular, it is preferable that the amino acid at the 3rd position is an L-amino acid or both the amino acids at the 3rd and 4th positions are L-amino acids.

[0152] The steric configuration of the amino acid residues selected at the 3rd and 4th positions can be suitably selected depending on the sequence of amino acids at 1st and 2nd positions. That is, both the 3rd and 4th positions residues are preferably L-amino acids in the case of the amino acid sequence Gly-Ser at the 1st and 2nd positions in natural ghrelin, whereas both the 3rd and 4th positions residues may be D-amino acids in the case of another amino acid sequence such as Aib-His. Further, if the residues at the 1st and 2nd positions are an  $\delta$ -amino acid (e.g., aminopentanoic acid) having a length of 2 amino acids, the residues at the 3rd and 4th positions may be L- or D-amino acids.

[0153] The amino acid residues selected at the 3rd and 4th positions are preferably D- or L-amino acids such as leucine, valine, norleucine, homoleucine, homoisoleucine, naphthyl alanine and its homologues thereof, tryptophan, phenylalanine and cyclohexyl alanine or D- or L-N-methylamino acids thereof.

[0154] The amino acid residues selected at the 3rd and 4th positions are more preferably aromatic hydrophobic amino acids such as naphthyl alanine and its homologues thereof, tryptophan, phenylalanine and cyclohexyl alanine, among the hydrophobic amino acids described above.

[0155] Further, the amino acid residues selected at the 3rd and 4th positions are preferably basic amino acids such as lysine, arginine and histidine. Especially, lysine is preferable.

[0156] The ghrelin molecule is to be basic by these basic amino acids, thus further improving the Ca-releasing activity.

[0157] The amino acid residues selected at the 3rd and 4th positions are preferably those having functional groups in their side chains, which can be modified with an acyl group (alkanyl group, alkenonyl group or aryl alkanyl group), an alkyl group or an aralkyl group, and preferable examples of such amino acids residues include serine, homoserine, threonine, cysteine, homocysteine, aspartic acid, glutamic acid, adipic acid, lysine, ornithine etc.

[0158] The amino acids having these reactive side chains may be either D- or L-amino acids or their corresponding D- or L-N-methyl amino acids, but it is particularly preferable that the 3rd position residue is an L-amino acid or both the 3rd and 4th positions residues are L-amino acids.

[0159] Further, to side chains of these amino acids may be bound an acyl group such as alkanyl group (the number of carbon atoms thereof is 2 to 35, preferably 6 to 18, more preferably 8 to 12), an alkenonyl group (the number of carbon atoms thereof is 2 to 35, preferably 6 to 18, more preferably 8 to 12), an aryl alkanyl group (benzoyl, phenacetyl, phenyl butyryl, naphthoyl, naphthyl acetyl or naphthyl propionyl group etc.), an alkyl group (the number of carbon atoms thereof is 2 to 35, preferably 6 to 18, more preferably 8 to 12), or an aralkyl group (benzyl, phenetyl, phenyl propyl, phenyl butyl, phenyl pentyl, naphthyl methyl group etc.) via a carbamate, thiocarbamate, ester, amide, disulfide, ether, thioether or thioester linkage. Further, the aforementioned alkyl and aralkyl groups may be bound not via the linkage to the  $\alpha$ -carbon atoms of amino acids at the 3rd and 4th positions.

[0160] The combination of amino acid residues selected at the 3rd and 4th positions is preferably a combination of an amino acid having a hydrophobic side chain as the 3rd position amino acid residue and a hydrophobic amino acid as the 4th position amino acid residue.

[0161] The 3rd position amino acid residue having a hydrophobic side chain is preferably a modified amino acid into the  $\alpha$  carbon of which (a) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced via or not via an alkylene group containing one or more carbon atoms and via an ester, ether, thioether, amide or disulfide linkage, or (b) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced. In particular, a modified amino acid into the  $\alpha$ -carbon of which a saturated alkyl chain containing one or more carbon atoms was introduced is more preferable.

[0162] The carboxyl group of amino acid at the 4th position may be an amide, an alkyl amide (e.g. methyl amide or ethyl amide), or an aralkyl amide (e.g. benzyl amide, adamantane amide or adamantane alkyl amide).

[0163] Further, a basic group such as amino group or guanidido group may be bound to the alkyl or aralkyl amide. The basic group includes e.g.  $-\text{CONH}-\text{CH}_2\text{CH}_2-\text{NH}_2$ ,  $-\text{CONH}-\text{CH}_2\text{NHCH}_3$ ,  $-\text{CONH}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}-\text{C}(\text{NH}_2)=\text{NH}$ , and  $-\text{CONHCH}_2\text{Ph}-\text{NH}_2$ .

[0164] A basic amino acid such as arginine, lysine and histidine may be added to the carboxyl group of amino acid at the 4th position, and this basic amino acid may be a D- or L-amino acid, a racemate, or D- or L-N-methyl amino acid.

[0165] The carboxyl group of the amino acid may be an alkyl or aralkyl amide as described above. Further, a basic group such as amino group or guanidido group may be added to the alkyl or aralkyl amide. The basic group includes those exemplified above.

[0166] As an amino acid sequence of amino acid at the 5th position and subsequent amino acids, a sequence of any length consisting of leucine at the 5th position and subsequent amino acids up to amino acid at the 28th position in human or rat ghrelin may be added to amino acid at the 4th position.

[0167] Such amino acid sequences are preferably ghrelin (1-5), ghrelin (1-6), ghrelin (1-7), ghrelin (1-8), ghrelin (1-9), ghrelin (1-10), and ghrelin (1-11) where ghrelin (m-n) refers to a peptide having an amino acid sequence at the m- to n-positions from the amino-terminal in ghrelin. In particular, ghrelin (1-5) is preferable.

[0168] The carboxyl-terminal thereof is preferably an alkyl or aralkyl amide as described above.

5 [0169] Further, a basic group such as amino group or guanidido group may be bound to the alkyl or aralkyl amide. The basic group includes those exemplified above.

[0170] Further, a basic amino acid such as arginine, lysine and histidine may be added to the carboxyl-terminal amino acid of a carboxyl-terminal-deleted ghrelin derivative wherein an amino acid sequence of any length consisting of amino acids from amino acid at 5th position up to amino acid at 28th position was added to the carboxyl-terminal of ghrelin  
10 (1-4).

[0171] This basic amino acid may be a D- or L-amino acid, a racemate, or D- or L-N-methyl amino acid.

[0172] The carboxyl group of the basic amino acid may be an alkyl or aralkyl amide as described above. Further, a basic group such as amino group or guanidido group may be bound to the alkyl or aralkyl amide. The basic group includes those exemplified above.

15 [0173] In a particularly preferable embodiment, the carboxyl-terminal amino acid of ghrelin (1-5), ghrelin (1-6) and ghrelin (1-7) is a D- or L-amino acid or its corresponding D- or L-N-methyl amino acid.

[0174] Further, basic amino acids such as arginine, lysine and histidine may be added to residues at the 5th, 6th and 7th position, and these basic amino acids may be D- or L-amino acids, racemates, or D- or L-N-methyl amino acids.

20 [0175] The carboxyl group of such a basic amino acid may be an alkyl or aralkyl amide as described above. Further, a basic group such as amino group or guanidido group may be bound to the alkyl or aralkyl amide. The basic group includes those exemplified above.

[0176] In a preferable embodiment in the present invention, the peptide compound of the present invention in the case where the carboxyl-terminal is an alkyl or aralkyl amide as described above may be an amide derivative wherein an amino group is further bound to the alkyl or aralkyl group. Specifically, a peptide compound wherein the carboxyl-terminal is e.g. aminoethyl amide can be mentioned.  
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[0177] The peptide-type compound of the present invention wherein the carboxyl-terminal is an amide or an amide derivative as described above is a useful compound because of its resistance to decomposition by enzymes such as carboxy peptidases *in vivo*.

30 [0178] Similarly, the peptide-type compound of the present invention including N-methyl amino acid is also a useful compound because of its resistance to enzymes.

[0179] The peptide-type compound of the present invention can be obtained in a usual manner. For example, it can be isolated from a natural source as mentioned above or produced by recombinant DNA technology and/or chemical synthesis. Further, when a modification (e.g., acylation) in the amino acid residues is necessary, the peptide compound can be subjected to a modification reaction by well-known methods in the art.

35 [0180] Specifically, the peptide-type compound of the present invention can be obtained by culturing host cells transformed with an expression vector harboring a DNA coding the peptide of the present invention and then recovering the desired peptide from the culture.

[0181] By selecting the host cells, a compound having the desired peptide modified by e.g. acylation in the cells can be obtained. When said peptide is not modified, a modification reaction such as acylation can be conducted as necessary by well-known methods in the art. For the acylation reaction, enzymes such as lipase can also be used.  
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[0182] The vector in which the gene is to be integrated includes e.g. *E. coli* vectors (pBR322, pUC18, pUC19 etc.), *Bacillus subtilis* vectors (pUB110, pTP5, pC194 etc.), yeast vectors (YE<sub>p</sub> type, YRp type, Ylp type), or animal cell vectors (retrovirus, vaccinia virus etc.), but any other vectors capable of maintaining the desired gene stably in host cells can also be used. The vector is introduced into suitable host cells. For integrating the desired gene into a plasmid and introducing the plasmid into host cells, methods described in Molecular Cloning (Sambrook et al., 1989) can be used.  
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[0183] To express the desired peptide gene in the above plasmid, a promoter is linked operatively upstream of said gene.

50 [0184] The promoter used in the present invention may be any suitable promoter compatible with host cells used for expression of the desired gene. For example, lac promoter, trp promoter, lpp promoter,  $\lambda$ PL promoter, recA promoter etc. can be used in the genus *Escherichia* as host cells to be transformed; SP01 promoter, SP02 promoter etc. can be used in the genus *Bacillus*; GAP promoter, PH05 promoter, ADH promoter etc. can be used in yeasts; and SV40-derived promoter, retrovirus-derived promoter etc. can be used in animal cells.

55 [0185] The desired gene-containing vector obtained in this manner is used to transform host cells. The host cells include microorganisms (for example, the genus *Escherichia*, the genus *Bacillus* etc.), yeast (the genus *Saccharomyces*, the genus *Pichia*, the genus *Candida* etc.), animal cells (CHO cells, COS cells etc.) etc. The medium for culture is preferably a liquid medium, and particularly preferably the medium containing a carbon source, a nitrogen source etc. necessary for growth of the transformed cells to be cultured. If desired, vitamins, growth promoters, serum etc.

can be added.

[0186] For directly producing the fatty acid-modified peptide, the cells are preferably those having the activity of a processing protease capable of cutting a suitable site in a precursor polypeptide of said peptide and the activity of acylating the serine residue in said peptide. Host cells having such processing protease activity and serine-acylating activity can be obtained by transforming host cells with an expression vector containing a cDNA coding said precursor polypeptide and then selecting the transformed cells by confirming whether or not they produce the fatty acid-modified peptide having a Ca-releasing activity or a GH secretion-inducing activity.

[0187] After culture, the peptide of the present invention is separated and purified from the culture in a usual manner. To extract the desired product from the cultured microorganisms or cells, for example, the microorganisms or cells after culture are collected and suspended in a buffer containing a protein denaturant (e.g. guanidine hydrochloride) and the microorganisms or cells are disrupted by sonication etc. and then centrifuged. To purify the desired product from the supernatant, separation and purification methods such as gel filtration, ultrafiltration, dialysis, SDS-PAGE, various chromatographic techniques can be suitably combined in consideration of the molecular weight, solubility, charge (isoelectric point), affinity etc. of the desired product.

[0188] The peptide compound of the present invention can be chemically synthesized in a usual manner. For example, amino acids having protective groups are condensed by a liquid phase method and/or a solid phase method to extend a peptide chain, then all protective groups are removed therefrom by an acid, and the resulting crude product is purified by the above purification techniques to give the desired peptide compound. An amino acid residue at the desired site can be acylated selectively by an acylase or acyl transferase.

[0189] Various methods have been well established for production of peptides, and the peptide-type compound of the present invention can also be easily produced by such known methods. For example, the peptide-type compound may be synthesized by the classical peptide synthesis method or by the solid phase method.

[0190] Hereinafter, a process for producing the peptide compound of the present invention by a combination of recombinant DNA technology and chemical synthesis is described by reference to examples.

[0191] Active esters of amino-terminal peptides, for example, (1) Boc-Gly-Ser(Bu)-Ser(R10)-Osu, (2) Boc-Gly-Ser(Bu)-Ser(R10)-Phe-Osu, and (3) Boc-Gly-Ser(Bu)-Ser(R10)-Phe-Leu-Osu, are chemically synthesized and then bound to carboxyl-terminal peptides produced by recombinant DNA technology, that is, (4) FLSPEHQVRVQQRKESKKPPAKLQPR, (5) LSPEHQVRVQQRKESKKPPAKLQPR, and (6) SPEHQVRVQQRKESKKPPAKLQPR, respectively; that is, (1) is bound to (4), (2) to (5), and (3) to (6), whereby peptide compounds each consisting of 28 amino acids are obtained respectively. Specifically, XXXXZSPEHQVRVQQRKESKKPPAKLQPR is expressed in *E. coli* followed by protection of its amino groups with Boc2(O) to give Boc-XXXXZSPEHQVRVQQRK(Boc)ESK(Boc)K(Boc)PPAK(Boc)LQPR. Then, the resulting peptide is converted into NH<sub>2</sub>-SPEHQVRVQQRK(Boc)ESK(Boc)K(Boc)PPAK(Boc)LQPR by cleavage with an enzyme selective for the carboxyl-terminal of amino acid Z. This compound is mixed with Boc-Gly-Ser(Bu)-Ser(R10)-Phe-Leu-Osu in an aqueous neutral to weak alkaline solution, and the resulting BocGlySer(Bu)Ser(R10)FLSPEHQVRVQQRK(Boc)ESK(Boc)K(Boc)PPAK(Boc)LQPR is treated with trifluoroacetic acid, whereby the desired product can be obtained.

[0192] The above one-letter notation of amino acid is in accordance with a description in Cellular Molecular Biology, 3rd edition published on December 10, 1997 by Newton Press Co., Ltd.

[0193] In addition, Boc represents t-butyloxycarbonyl, Osu represents a residue derived from N-hydroxysuccinimide by eliminating hydrogen from the hydroxyl group thereof, Bu represents a butyl group, and R10 represents the substituent group of the modified amino acid according to the present invention.

[0194] Salts of the peptide-type compound of the present invention are preferably pharmaceutically acceptable salts including, for example, salts with inorganic bases, salts with organic bases, salts with inorganic acids, salts with organic acids, and salts with basic or acidic amino acids.

[0195] Preferable examples of the salts with inorganic bases include alkali metal salts such as sodium salts, potassium salts etc.; alkaline earth metal salts such as calcium salts, magnesium salts etc.; and aluminum salts, ammonium salts etc.

[0196] Preferable examples of the salts with organic bases include salts with trimethylamine, triethylamine, pyridine, picoline, ethanolamine, diethanolamine, triethanolamine, dicyclohexyl amine, N,N'-dibenzylethylene diamine etc.

[0197] Preferable examples of the salts with inorganic acids include salts with hydrochloric acid, hydrobromic acid, nitric acid, sulfuric acid, phosphoric acid etc.

[0198] Preferable examples of the salts with organic acids include salts with formic acid, acetic acid, trifluoroacetic acid, fumaric acid, oxalic acid, tartaric acid, maleic acid, citric acid, succinic acid, malic acid, methanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid etc.

[0199] Preferable examples of the salts with basic amino acids include salts with arginine, lysine, ornithine etc., and suitable examples of the salts with acidic amino acids include salts with aspartic acid, glutamic acid etc.

[0200] Among these salts, sodium salts and potassium salts are most preferable.

[0201] The peptide-type compound of the present invention or pharmaceutically acceptable salts thereof are low



toxic and have a GH-secretion inducing action, and they can be administered as such or after mixed with known, pharmaceutically acceptable carriers, excipients, vehicles augmentors etc., to a mammal (for example, human being, mouse, rat, rabbit, dog, cat, bovine, horse, porcine, monkey etc.). In the case of intravenous injection into an adult, the daily dose is 0.01 to 5 mg/kg, preferably 0.04 to 1.5 mg/kg. This dose is administered desirably once to thrice every day. The peptide-type compound of the present invention is compounded with pharmaceutically acceptable carriers and can be orally or parenterally as solid pharmaceutical preparations such as tablets, capsules, granules, powders etc. or as liquid pharmaceutical preparations such as syrups, injections etc.

[0202] The pharmaceutically acceptable carriers include a wide variety of organic or inorganic carriers which are customarily used as pharmaceutical materials, and these are compounded as vehicles, lubricants, binders, disintegrant in solid pharmaceutical preparations or as solvents, adjuvant, suspending agents, isotonicity-conferring agents, buffers and soothing agents in liquid pharmaceutical preparations.

[0203] As necessary, pharmaceutical additives such as preservatives, antioxidants, coloring agents, sweeteners etc. can also be used.

[0204] Preferable examples of the vehicles include e.g. lactose, white sugar, D-mannitol, starch, crystalline cellulose, light anhydrous silicic acid etc. Preferable examples of the lubricants include magnesium stearate, calcium stearate, talc, colloidal silica etc.

[0205] Preferable examples of the binders include crystalline cellulose, white sugar, D-mannitol, dextrin, hydroxypropyl cellulose, hydroxypropylmethyl cellulose, polyvinyl pyrrolidone etc.

[0206] Preferable examples of the disintegrant include starch, carboxymethyl cellulose, carboxymethyl cellulose calcium, croscarmellose sodium, carboxymethyl starch sodium etc.

[0207] Preferable examples of the solvents include water for injection, alcohol, propylene glycol, macrogol, sesame oil, corn oil etc.

[0208] Preferable examples of the solubilizers include polyethylene glycol, propylene glycol, D-mannitol, benzyl benzoate, ethanol, trisaminomethane, cholesterol, triethanolamine, sodium carbonate, sodium citrate etc.

[0209] Preferable examples of the suspending agents include surfactants such as stearyl triethanolamine, sodium lauryl sulfate, lauryl aminopropionic acid, lecithin, benzalconium chloride, benzethonium chloride and glycerin monostearate, and hydrophilic polymers such as polyvinyl alcohol, polyvinyl pyrrolidone, carboxymethyl cellulose sodium, methyl cellulose, hydroxymethyl cellulose, hydroxyethyl cellulose and hydroxypropyl cellulose.

[0210] Preferable examples of the isotonicity-conferring agents include sodium chloride, glycerine, D-mannitol etc.

[0211] Preferable examples of the buffers include buffer solutions such as phosphates, acetates, carbonates, citrates etc.

[0212] Preferable examples of the soothing agents include benzyl alcohol etc.

[0213] Preferable examples of the preservatives include p-oxyesterbenzoates, chlorobutanol, benzyl alcohol, phenetyl alcohol, dehydroacetic acid, sorbic acid etc.

[0214] Preferable examples of the antioxidants include sulfites, ascorbic acid etc.

[0215] The above pharmaceutical composition brings about an effect equal to or higher than the effect of GH upon administration and can reduce various side effects caused by administration of GH.

[0216] As diseases attributable to deficiency or reduction in GH, the diseases to which the pharmaceutical composition can be applied or the effects of the pharmaceutical composition include, but are not limited to, activation of osteoblasts and re-constitution of bone in people with dwarfism and normal human beings, enhancement of muscular strength and muscular amount in GH-deficient adults, improvement of motility in GH-deficient adults, remedy of heavy burns in children, its combined use with gonadotropins in induction of ovulation, prevention of abnormalities in protein metabolism by administration of prednisone, promotion of T cell "education" in heavy immune disorder, the effect of inhibiting reduction of the body weight of the aged and the effect of enlarging adipose tissues and preventing dermal atrophy.

[0217] Further, the diseases or effects not directly correlated with deficiency or reduction in GH include e.g. the effect of increasing pulsatile flow as shown in Example 7, and thus it is effective for treatment of cardiac diseases such as cardiac failure etc.

[0218] The effect of the pharmaceutical composition is not restricted to human beings. That is, it has an effect on growth promotion for animals, reduction of fat in meat, etc., which is equal to or higher than administered GH.

[0219] For example, as shown in Example 13, the pharmaceutical composition of the present invention exhibits an appetite-promoting action upon administration into ventricle or intravenous administration, so it can be used as an appetite promoter for treating anorexia or sitophobia.

[0220] In addition, as shown in Example 14, the pharmaceutical composition of the present invention has a stomach motility- and gastric acid secretion-promoting action, and thus it can also be used as an agent for treating stomach functional diseases such as non-diabrotic dyspepsia, sudden light stomach atony, functional dyspepsia, and reflux esophagitis.

[0221] Further, as shown for example in Example 15, the pharmaceutical composition of the present invention exhibits

a cell growth-promoting action in bone marrow, duodenum and jejunum by intravenous administration, and thus it can be used as an agent for protecting small intestine tunica mucosa, an agent for preventing damage to small intestine tunica mucosa during intravenous nutrition and an agent for treating osteoporosis.

[0222] Further, the pharmaceutical composition described above has an effect for treating the diseases or for improving the physical conditions described below.

[0223] For example, it can be used for stimulative treatment for release of growth hormones in the aged, prevention of catabolic side effects of sugar corticoids, prevention and treatment of osteoporosis, stimulation at immune system, promotion of remedy of damages, promotion of repair of broken bone, treatment of growth delay, treatment of renal insufficiency or functional insufficiency attributable to growth delay, treatment of insufficient conditions correlated with physiological insufficient conditions including growth hormone-deficient children and chronic diseases, treatment of obesity and growth delay correlated with obesity, treatment of growth delay correlated with Plauda-Villi syndrome and Taner syndrome, promotion of recovery of burn patient and reduction in hospitalization, treatment of intrauterine growth delay, skeleton mulformation, hypercorticotid disease and Cusshing syndrome, induction of release of pulsatile growth hormone, replacement of growth hormone in stressed patients, cartilage mulformation, Noonan syndrome, schizophrenia, depression, Alzheimer's disease, remedy of delayed repair of damage and physicosocial deprivation, treatment of pulmonary insufficiency and respiratory organ dependence, decrease of catabolic reaction of protein after major operation, reduction of protein loss and cachexia caused by chronic diseases such as cancer and AIDS, treatment of hyperinsulinism including pancreas nesidioblastosis, adjuvant therapy for induction of ovulation, and treatment of patients with immune repression, improvement of muscular strength and motility, maintenance of thickness of skin in the aged, metabolic homeostasis and renal homeostasis, stimulation of osteoblast, re-formation of bone and stimulation of cartilage growth, in order to stimulate growth of thymus and to prevent deterioration in thymic functions accompanying aging.

[0224] Further, the following effects on animals are also expected. For example, mention is made of an increase in the rate of animal growth, an increase in production of milk and fur in animals, stimulation of the immune system in pet animals, treatment of diseases caused by advanced age in pet animals, growth promotion of domestic animals, and an increase in fur in sheep.

[0225] An antibody whose antigen is fatty acid-modified peptide of the present invention having a Ca-releasing activity or a GH secretion-inducing activity can be obtained by a method known in the art. The antibody may be a monoclonal or polyclonal antibody, and can be obtained by a method known in the art. Further, a method for measuring the fatty acid-modified peptide using said antibody and a measuring kit using said measuring method can also make use of a method known in the art.

[0226] As described in Example 17, antibodies to amino- and carboxyl-terminal peptides from ghrelin are prepared respectively, and since the former recognizes fatty acid-modified serine at the 3rd position, both the antibodies can be used to separate and quantify ghrelin modified with a fatty acid and ghrelin from which the fatty acid was eliminated.

[0227] The antibodies to amino- and carboxyl-terminal peptides in ghrelin can be obtained in a known method, and they may be monoclonal or polyclonal antibodies.

[0228] For the present peptide-type compound having a modified amino acid at the 3rd position from the amino-terminal or a pharmaceutically acceptable salt thereof, an antibody which specifically recognizes a side chain of 3rd amino acid residue (preferably a fatty acid) and binds to an amino-terminal partial peptide of the peptide-type compound can also be produced in the same manner. In addition, for the peptide-type compound of the present invention or a pharmaceutically acceptable salt thereof, an antibody which binds specifically to the peptide having a modified amino acid can also be produced in the same manner.

[0229] The present invention also encompasses an examination kit comprising a combination of an antibody specifically recognizing a side chain of the modified amino acid and an antibody recognizing amino acids (or a peptide) excluding the modified amino acid and/or a non-amino acid compound, preferably an antibody to a carboxyl-terminal partial peptide of the peptide-type compound of the present invention or of a pharmaceutically acceptable salt thereof, as described above.

[0230] Further, the present invention encompasses an assay method wherein the peptide-type compound of the present invention having a modified amino acid, preferably an acylated amino acid, or a pharmaceutically acceptable salt thereof, and the peptide-type compound of the present invention not containing a modified amino acid, or a pharmaceutically acceptable salt thereof, are separated and detected by use of said examination kit.

[0231] Hereinafter, the assay method and examination kit described above are described by reference to their embodiments, which however are not intended to limit the present invention.

[0232] That is, the assay method includes, for example, (i) a method of quantifying the peptide-type compound etc. of the present invention in a test solution, which comprises allowing a test material in a test solution and the labeled peptide-type compound etc. of the present invention to react competitively with an antibody to the peptide-type compound etc. of the present invention and then determining the ratio of the labeled peptide-type compound etc. of the present invention bound to said antibody, and (ii) a method of quantifying the proteins etc. of the present invention in

a test solution, which comprises allowing a test solution to react with the antibody of the present invention insolubilized on a carrier and another labeled antibody of the present invention simultaneously or successively and then measuring the activity of the labeling agent on the insolubilizing carrier and/or the activity of the labeling agent not captured on the insolubilizing carrier. In the quantification methods (i) and (ii), it is preferable that one antibody is an antibody recognizing an amino-terminal region of the protein etc. of the present invention, while the other antibody is an antibody reacting to a carboxyl-terminal region of the protein etc. of the present invention.

[0233] In the method of assaying the peptide-type compound etc. of the present invention, the monoclonal antibody against said compound (also referred hereinafter to as the anti-protein antibody) can be used not only for quantifying the protein etc. of the present invention, but also for detection thereof by tissue staining etc.

[0234] For these purposes, either the antibody molecule itself or an F(ab')<sub>2</sub>, Fab' or Fab fraction of the antibody molecule may be used.

[0235] The method of quantifying the peptide-type compound etc. of the present invention by use of said antibody is not particularly limited as long as the method comprises detecting the amount of the antibody corresponding to the amount of the antigen (e.g., the amount of the protein), the antigen or an antigen-antibody conjugate in a test solution, by chemical or physical means and calculating it on the basis of a standard curve prepared using standard solutions containing known amounts of the antigen. For example, nephelometry, the competitive method, the immunometric method and the sandwich method are preferably used, but the sandwich method described later is particularly preferably used in respect of sensitivity and specificity.

[0236] For a measurement method using a label in the assay method of the present invention, the label includes e.g. a radioisotope, an enzyme, a fluorescent material and a luminescent material.

[0237] The radioisotope includes, for example, <sup>125</sup>I, <sup>131</sup>I, <sup>3</sup>H, <sup>14</sup>C etc.

[0238] The enzyme is preferably a stable enzyme with high specific activity, which includes, for example,  $\beta$ -galactosidase,  $\beta$ -glucosidase, alkali phosphatase, peroxidase and malate dehydrogenase.

[0239] The fluorescent material includes, for example, fluorescamine and fluorescein isocyanate.

[0240] The luminescent material includes, for example, luminol, luminol derivatives, luciferin, and lucigenin.

[0241] Further, a biotin-avidin system can also be used for binding the label to the antibody or antigen.

[0242] Hereinafter, the present invention is described in more detail by reference to the Examples. Unless otherwise specified, the genetic manipulation means were in accordance with Molecular Cloning (Sambrook et al., 1989).

#### Example 1. Creation of a cell strain expressing GHS-R and measurement of Ca-releasing activity.

[0243] To assay an increase in the intracellular calcium ion concentration (Ca-releasing activity) occurring upon binding of a GH secretagogue (GHS) to GHS-receptor (GHS-R), a cell strain expressing rat GHS-R was created in the following manner. A full-length cDNA for rat GHS-R was obtained by RT-PCR (reverse transcriptase-polymerase chain reaction) where a cDNA derived from rat brain was used as template. From the nucleotide sequence of known rat GHS-R [K. K. McKee, et al, Molecular Endocrinology 11, 415-423 (1997)], sense and antisense primers consisting of the following nucleotide sequences were synthesized.

**Sense primer: 5' -ATGTGGAACGCGACCCCCAGCGA-3'**

**Antisense primer: 5' -ACCCCCAATTGTTTCCAGACCCAT-3'**

[0244] The amplified cDNA was ligated to vector pcDNAIII (Invitrogen) to construct expression vector GHSR-pCDNAIII. CHO cells were transformed via the expression vector, and the transformed cells stably expressing GHS-R were selected in a medium containing 1  $\mu$ g/ml G418. The selected cell strain CHO-GHSR62 responded to 10<sup>-10</sup> to 10<sup>-9</sup> M GHRP-6 (Growth Hormone-Releasing hexapeptide). A change in the intracellular calcium ion concentration (Ca-releasing activity) was measured by an FLIPR system (Molecular Device). Before this measurement, 4 $\times$ 10<sup>4</sup> CHO-GHSR62 cells were put to a 96-well microplate with black wall (Corning Co., Ltd) and cultured for 12 to 15 hours. Then, the cells were incubated with 4  $\mu$ M fluorescent Fluo4 (Molecular Probe Co., Ltd) for 1 hour and washed four times with Hank's BSS (Hank's Balanced Salt Solution) containing 20 mM Hepes ([N-2-hydroxyethyl]-piperazine-N-[2-ethanesulfonic acid]) and 2.5 mM probenecid, and the Ca-releasing activity was assayed by adding a sample and measuring a change in the fluorescence.

#### Example 2. Purification of an endogenous GH secretion-inducing peptide

[0245] Using the CHO-GHSR62 cells described in Example 1, a wide variety of rat-derived tissues and organs were

examined for their Ca-releasing activity, and as a result, it was found that a peptide extract derived from rat stomach has a strong Ca-releasing activity even in a small amount of 0.5 mg. Accordingly, a peptide having the Ca-releasing activity was purified from a rat stomach extract by several kinds of chromatography.

[0246] 40 g fresh rat stomach was boiled for 5 minutes in 5-fold boiling water to inactivate proteases present in it. After cooling, the boiled sample was placed in 1M AcOH-20 mM HCl, followed by extracting the peptide by a Polytron mixer. The extract was centrifuged at 11,000 rpm for 30 min., and the supernatant was concentrated into about 40 ml in an evaporator. The concentrate was precipitated with acetone by adding acetone thereto at a concentration of 66 %, and after the formed precipitates were removed, the acetone in the supernatant was evaporated. The supernatant was applied to 10 g Sep-Pak C18 cartridge (Waters Co.,Ltd) previously equilibrated with 0.1% TFA (trifluoroacetic acid), washed with 10 % CH<sub>3</sub>CN/0.1% TFA and then eluted with 60 % CH<sub>3</sub>CN/0.1% TFA. After the solvent in the eluate was evaporated, the sample was lyophilized. The lyophilized sample was dissolved in 1 M AcOH and absorbed onto SP-Sephadex C-25 (H<sup>+</sup> type) previously equilibrated with 1 M AcOH. The sample was eluted stepwise with 1 M AcOH, then with 2 M pyridine, and finally with 2 M pyridine-AcOH (pH 5.0), whereby 3 fractions, that is, SP-I, SP-II and SP-III were obtained respectively. The SP-III fraction was applied to a Sephadex G-50 gel filtration column, and an aliquot of each fraction was assayed for Ca-releasing activity by use of the CHO-GHSR62 cells. A profile in Sephadex G-50 column chromatography is shown in Fig. 1a, and active fractions (fractions 43 to 48 in Fig. 1a) having molecular weights of about 3,000 were fractionated by high performance liquid chromatography (HPLC) by CM-ion exchange on TSK CM-2SW column (4.6×250 mm, Tosoh Corp.) at pH 6.4. The active fractions by CM-HPLC were secondarily fractionated by CM-HPLC on the same column at pH 4.8 (Fig. 1b). The active fractions (elution time of 55 to 56 minutes in Fig. 1b) were purified to homogeneity by reverse phase HPLC on  $\mu$ Bondasphere C-18 column (3.9×150 mm, Waters Co.,Ltd). From 40 g of the rat stomach, 16  $\mu$ g peptide having a Ca-releasing activity was purified and designated as ghrelin.

### Example 3. Structural analysis of ghrelin

[0247] The amino acid sequence of the purified ghrelin derived from rat was determined by a peptide sequencer (ABI 494, Applied Biosystems Co.,Ltd). The ghrelin was a peptide composed of 28 amino acid residues consisting of the following sequence: Gly Ser Xaa Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg where Xaa is an unidentified amino acid. On the basis of the nucleotide sequence of the rat cDNA, Xaa was estimated to be Ser, indicating a certain modification on Ser in the peptide.

[0248] Accordingly, unmodified ghrelin wherein serine at the 3rd position from the amino-terminal was chemically synthesized by a peptide synthesizer (ABI 433A, Applied Biosystems Co.,Ltd). Because the elution time of the unmodified synthetic ghrelin in reverse phase HPLC was significantly different from that of natural ghrelin (Fig. 2a), the unmodified synthetic ghrelin was found to be more significantly hydrophilic than natural ghrelin.

[0249] From the above result, it was found that in natural ghrelin, serine at the 3rd position from the amino-terminal (3rd serine) has been modified with a hydrophobic residue.

[0250] To reveal the modifying group on 3rd serine, the purified ghrelin was analyzed by electrospray ionization mass spectrometry (ESI-MS). The found molecular weight ( $3314.9 \pm 0.7$ ) of natural ghrelin was greater by about 126 than the molecular weight (3188.5) of the unmodified ghrelin peptide which was estimated from the nucleotide sequence of the cDNA. From the above result, it was found that the hydroxyl group of 3rd serine in natural ghrelin has been modified with n-octanoyl (C8 : 0) fatty acid.

[0251] To confirm this, the n-octanoyl (C 8 : 0) ghrelin peptide was chemically synthesized and examined for its elution time in reverse phase HPLC. In chemical synthesis of the n-octanoyl (C8 : 0) peptide, the peptide wherein all functional groups except for the hydroxyl group of 3rd serine were protected was synthesized by the Fmoc solid phase method using a peptide synthesizer (ABI 433A, Applied Biosystems Co.,Ltd), and then the desired peptide was obtained by acylation of the hydroxyl group of 3rd serine with n-octanoic acid and ethyl-3-(3-dimethylaminopropyl)carbodiimide in the presence of 4-(dimethylamino)pyridine. The synthetic n-octanoyl peptide indicated the same elution time as that of the purified natural ghrelin (Fig. 2a). Further, the synthetic n-octanoyl peptide and a peptide fragment at the 1st to 4th positions from the amino-terminal (Gly 1-Phe 4) which was obtained by treating natural ghrelin with chymotrypsin showed the same retention time in reverse phase HPLC.

[0252] From the above result, it was concluded that the natural ghrelin derived from rat has the amino acid sequence set forth in SEQ ID NO: 2, wherein the hydroxyl group of 3rd serine has been acylated with n-octanoic acid (caprylic acid) (Fig. 2c).

[0253] Further, human ghrelin was purified from a human stomach extract, and it was found that its structure has the amino acid sequence set forth in SEQ ID NO: 3, wherein the side-chain hydroxyl group of 3rd serine has been acylated with n-octanoic acid (caprylic acid) (Fig. 4a).

[0254] The structures of the rat- and human-derived ghrilins were determined using those purified as the first-peak fractions (elution time of 55 to 56 minutes) out of the active fractions in Fig. 1b, and after purification, the structure of the other active fractions in Fig. 1b was also analyzed in the same manner, indicating the presence of not only caprylic

acid (C8 : 0) but also its monoene acid (C8 : 1), capric acid (C10 : 0) and its monoene acid (C10 : 1), and lauric acid (C12 : 0) and its monoene acid (C12 : 1) as the modifying fatty acid on 3rd serine.

[0255] Further, chicken, eel and frog ghrelins were purified from stomach extracts in the same manner as in Example 2 and analyzed for their structure in the same manner as in Example 3. It was found that the chicken ghrelin has the amino acid sequence shown in SEQ ID NO: 25, the eel ghrelin has the amino acid sequence shown in SEQ ID NO: 26, and the frog ghrelin has the amino acid sequence shown in SEQ ID NO: 27, and in all of the ghrelins, the side-chain hydroxyl group of 3rd serine has been acylated with n-octanoic acid (caprylic acid).

[0256] Further, frog (*Xenopus Laevis*), fish (rainbow trout) and canine ghrelins were purified from stomach extracts in the same manner as in Example 2 and analyzed for their structure in the same manner as in Example 3.

[0257] It was found that the frog ghrelin has the amino acid sequence shown in SEQ ID NO: 28, the rainbow trout ghrelin has the amino acid sequence shown in SEQ ID NO: 29 and 30, and the canine ghrelin has the amino acid sequence shown in SEQ ID NO: 31, and in all of the ghrelins, the side-chain hydroxyl group of 3rd serine or threonine has been acylated with n-octanoic acid (caprylic acid).

[0258] From the rainbow trout, ghrelin-23 consisting of 23 amino acid residues shown in SEQ ID NO: 29 and ghrelin-20 consisting of 20 amino acid residues shown in SEQ ID NO: 30 were obtained.

#### Example 4. Ca-releasing activity of ghrelin.

[0259] The natural ghrelin and n-octanoyl-modified synthetic ghrelin had a Ca-releasing activity, but the unmodified synthetic ghrelin did not show a significant Ca-releasing activity (Fig. 2b). Further, because n-octanoic acid or amixture of n-octanoic acid and the unmodified synthetic ghrelin did not show a significant Ca-releasing activity, it was found that the n-octanoic acid residue in the natural ghrelin constitutes an important structure for the Ca-releasing activity. Hereinafter, ghrelin refers to [O-n-octanoyl-serine 3]-ghrelin (Fig. 2c).

[0260] In CHO-GHSR62 cells, the ghrelin exhibited a higher activity of increasing the intracellular calcium ion concentration (Ca-releasing activity) than that by GHRP-6, while GHRH (GH releasing hormone, expressed as GRF in Fig. 3a) did not exhibit the Ca-releasing activity (Fig. 3b). The Ca-releasing activity of ghrelin was recognized at a concentration of  $10^{-11}$  M or more, and its  $EC_{50}$  was 2.5 nM. The Ca-releasing activity of ghrelin was inhibited in the presence of  $10^{-4}$  M specific antagonist ([D-Lys 3]-GHRP-6) for GHS-R [R. G. Smith, et al., Science 260, 1640-1643 (1993)], and the Ca-releasing activity was restored at a high concentration of ghrelin in the absence of the antagonist (Fig. 3b). The above result indicates that the Ca-releasing activity of ghrelin is inhibited antagonistically by the specific antagonist for GHS-R.

#### Example 5. A cDNA for a ghrelin precursor and expression thereof in various organs

[0261] The amino acid sequence of the ghrelin had no homology with the amino acid sequences of any known peptides, but as a result of homology examination in the GenBank data base, the same sequence was found in a rat EST (Expressed Sequence Tag) sequence (GenBank acceptance No. A1549172). On the basis of this EST sequence, the following PCR primers were synthesized:

**Sense primer: 5' -TTGAGCCCAGAGCACCAGAAA-3'**

**Antisense primer: 5' -AGTTGCAGAGGAGGCAGAAGCT-3'**

[0262] These 2 primers were used in RT-PCR where a rat stomach-derived cDNA was used as template. PCR conditions utilized 35 cycles each consisting of 98 °C for 10 seconds, 55 °C for 30 seconds and 72 °C for 1 minute. The amplified DNA fragment was used as a probe for screening a rat stomach cDNA library. By screening about  $2 \times 10^5$  recombinant phages, a full-length cDNA coding the rat-derived ghrelin was obtained.

[0263] The rat ghrelin cDNA was composed of 501 bases shown in SEQ ID NO: 6, coding a ghrelin precursor (prepro-ghrelin) consisting of 117 amino acids (Fig. 4a). The amino-terminal 23 amino acid residues in the ghrelin precursor had properties as a signal peptide. The ghrelin starts from glycine 24, and the last 2 amino acids (Pro-Arg) in the mature ghrelin was a sequence undergoing cleavage with a protease.

[0264] Using the rat ghrelin cDNA, a human stomach cDNA library was screened under low-stringency conditions to obtain a full-length human ghrelin cDNA. The human stomach cDNA library was prepared from human gastric poly (A)<sup>+</sup>RNA (Clontech Co.,Ltd) by use of a cDNA synthesis kit (Pharmacia Co.,Ltd). The full-length human ghrelin cDNA thus obtained was composed of 511 bases shown in SEQ ID NO: 7, coding a human ghrelin precursor (prepro-ghrelin) consisting of 117 amino acids (Fig. 4a). Homology at the amino acid sequence level between the rat- and human-

derived ghrelin precursors was 82.9 %, revealing that ghrelins are highly conserved between species.

[0265] To know the distribution of ghrelin in tissues, poly(A)+RNA isolated from various rat tissues was analyzed (Fig. 4b). By Northern blotting analysis of the rat tissues, 0.62kb ghrelin precursor mRNA was recognized in stomach. Two faint bands were also recognized in ventricle, and these were 6.2kb and 1.2 kb mRNAs which were larger than the mRNA in stomach, thus suggesting different mRNA splicing from that in stomach. From the above result, it was found that a major expression site for ghrelin is stomach.

#### Example 6. Effect of ghrelin on secretion of pituitary hormones

[0266] Whether ghrelin has GH secretion-inducing activity or not was examined *in vitro* and *in vivo*. First, the effect of ghrelin on primary cultured cells of anterior pituitary was examined for assay *in vitro*. Anterior pituitaries were collected from 4-week-old male SD rats and dispersed by treatment with collagenase, and the cells were collected, washed twice with DMEM medium (Dulbecco's modified Eagle's medium) containing 10 % FCS (fetal bovine serum) and an antibiotic, and suspended in DMEM medium to prepare primary cultured cells of anterior pituitary. The  $5 \times 10^4$  cells were inoculated onto a 96-well cell culture plate coated with poly-D-lysine, and cultured for 3 to 4 days. The culture medium was exchanged with DMEM medium containing 0.1 ml sample, and maintained at 37°C for 15 minutes. An aliquot of the culture medium was collected and measured by radioimmunoassay for the concentrations of various pituitary hormones in the culture medium. Among the pituitary hormones, GH, FSH, LH, PRL and TSH were measured by using a kit produced by Biotrak/Amersham Co., Ltd, and ACTH was measured by using a high-sensitivity EIA kit produced by Peninsula Laboratories.

[0267] When ghrelin was added to primary cultured cells of anterior pituitary, an increase in the intracellular calcium ion concentration was recognized, while unmodified synthetic ghrelin also showed a slightly increased Ca-releasing activity (Fig. 5a). This result indicates that both ghrelin and unmodified ghrelin act directly on pituitary cells. Then, the GH secretion-inducing activity of ghrelin was examined using the primary cultured cells of anterior pituitary, and by addition of  $10^{-6}$  M ghrelin, the concentration of GH in the culture was increased depending on concentration, but no increase in the concentrations of other pituitary hormones (FSH, LH, PRL, TSH) was observed (Fig. 5b).

[0268] The GH secretion-inducing activity of ghrelin was examined *in vivo*. After 10 µg of synthetic ghrelin was injected intravenously into a male rat (250 g), the blood was collected periodically for up to 60 minutes to measure the concentration of the pituitary hormones in plasma by radioimmunoassay. Among the pituitary hormones, only GH was released into blood and reached the maximum level in 5 to 10 minutes after intravenous injection of ghrelin. From this result, it was found that ghrelin released from stomach to blood act on cells of anterior pituitary and release GH into blood, and it was confirmed that the ghrelin is an unidentified specific endogenous GH-secretion-inducing substance.

#### Example 7. Increase in cardiac output in rat

[0269] The effect of acute administration of ghrelin into a rat under anesthesia on the cardiovascular system was examined. Wistar male rats (Carerle) each weighing 220 to 250 g were divided at random into 4 groups (as groups given 10, 1, 0.5, and 0.2 µg ghrelin respectively) in order to examine the effect of acute administration of ghrelin on the cardiovascular system. Ghrelin was diluted with physiological saline and then prepared a dose of 10, 1, 0.5, or 0.2 µg/rat, and rapidly administered 120 µl via an injection tube (PE50) which had been inserted into the right common jugular vein for measuring cardiac output.

[0270] As a dynamic indicator, systemic blood pressure and cardiac output were measured, and peripheral vascular resistance was calculated. The rats were anesthetized with pentobarbital and fixed with dorsal position. For measurement of average blood pressure, a polyethylene canula (PE50) filled with heparin was inserted into the right femoral artery. The cardiac output was measured using a thermal dilution-type cardiac output meter (CARDIOTHER M500R). An injection tube (PE50) filled with physiological saline was inserted into the right common jugular vein in the rat and retained in the right ventricle. A micro-catheter was inserted into from the right common jugular vein in the rat and retained in the initiating part of the aorta. The infusion made use of 100 µl physiological saline at room temperature (25 °C). By pushing a MEASURE switch of the thermal dilution-type cardiac output meter and simultaneously injecting the infusion (100 µl physiological saline), measurement of cardiac output was initiated. Cardiac output was measured 5 times to determine average cardiac output. Average blood pressure and cardiac output were determined in 1, 5, 15, and 30 minutes before and after administration of ghrelin. Peripheral vascular resistance was determined by dividing average blood pressure by cardiac output.

Table 1

	Body weight (g)	Cardiac output (ml/min/kg) after administration of 1 µg ghrelin				
		0 min.	1 min.	5 min.	15 min.	30 min.
Mean	230	347	382	367	341	338
SEM	3.7	14.3	10.2	11.5	7.9	8.8

[0271] In the table, SEM is standard error means.

Table 2

	Body weight (g)	Cardiac output (ml/min/kg) after administration of 10 µg ghrelin				
		0 min.	1 min.	5 min.	15 min.	30 min.
Mean	237	350	390	392	370	344
SEM	1.0	8.5	7.4	15.8	14.7	13.8

[0272] In the Table, SEM is standard error means.

[0273] In the group given 1 µg ghrelin (Table 1) and the group given 10 µg ghrelin (Table 2), an increase in cardiac output was recognized in 1 to 5 minutes after administration.

#### Example 8. Isolation of ghrelin and ghrelin-27 from various origins

[0274] From rat stomach extract, ghrelin was purified using Ca-releasing activity as an indicator in the method described in Example 2. The active fraction (elution time of 59 minutes in Fig. 1b) in secondary CM-HPLC was purified to homogeneity by reverse phase HPLC on µBondasphere C-18 column (3.9×150 mm, produced by Waters Co., Ltd). This fraction was analyzed by electrospray ionization mass spectrometry (ESI-MS), indicating a peak of the molecular weight (3187.2±0.9) which was smaller by about 126 than that of natural ghrelin modified with octanoic acid (C8) consisting of 28 amino acids. Determination of the amino acid sequence of this peptide by a peptide sequencer (ABI 494, manufactured by Applied Biosystems Co., Ltd) revealed that it is a peptide composed of the following 27 amino acid residues: Gly Ser Xaa Phe Leu Ser Pro Glu His Gln Lys Ala Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg (Xaa is an unidentified amino acid). That is, this peptide was composed of an amino acid sequence wherein in ghrelin consisting of 28 amino acids, 13th or 14th glutamine was deleted. Since the Ca-releasing activity of this peptide was similar to that of ghrelin of 28 amino acids as shown in Example 9, this peptide was designated as ghrelin-27. From human stomach extract, human ghrelin-27 was isolated in the same manner as for rat ghrelin, and confirmed to consist of the amino acid sequence set forth in SEQ ID NO: 11. Peak fractions with retention times of 64 to 65 minutes in secondary CM-HPLC were purified and analyzed by electrospray ionization mass spectrometry (ESI-MS), indicating a peak of the molecular weight (3341.4±0.9). Because this fatty acid-modified peptide was composed of 28 amino acids, it was revealed to be a peptide wherein in ghrelin (28 amino acids), 3rd serine was modified with decanoic acid (C10).

[0275] From the rat stomach cDNA library prepared in Example 5, a cDNA coding a precursor of ghrelin-27 was cloned by plaque hybridization where the PCR-amplified DNA fragment prepared in Example 5 was used as a probe. The nucleotide sequence of the cDNA was determined and confirmed to code the precursor of ghrelin-27. The resulting cDNA for the rat ghrelin-27 precursor was composed of the nucleotide sequence set forth in SEQ ID NO: 14, coding the ghrelin-27 precursor having the amino acid sequence (116 amino acids) set forth in SEQ ID NO: 12. A cDNA for the human ghrelin-27 precursor was also cloned in the same manner as described above, and revealed to consist of the nucleotide sequence set forth in SEQ ID NO: 15, coding the human ghrelin-27 precursor having the amino acid sequence (116 amino acids) set forth in SEQ ID NO: 13.

[0276] A cDNA coding a precursor of porcine-derived ghrelin or ghrelin-27 was cloned from a porcine cDNA library in the method described in Example 5 by plaque hybridization where the PCR-amplified DNA fragment described in Example 5 was used as a probe. The nucleotide sequence of the resulting cDNA clone was determined and confirmed to code a porcine ghrelin precursor or a porcine ghrelin-27 precursor. The resulting cDNA for the porcine ghrelin precursor was composed of the nucleotide sequence set forth in SEQ ID NO: 20, coding a ghrelin precursor having the amino acid sequence (118 amino acids) set forth in SEQ ID NO: 18. The cDNA for the porcine ghrelin-27 precursor was composed of the nucleotide sequence set forth in SEQ ID NO: 21, coding the ghrelin-27 precursor having the amino acid sequence (117 amino acids) set forth in SEQ ID NO: 19. Accordingly, the porcine ghrelin (28 amino acids)

and the porcine ghrelin-27 (27 amino acids) are composed of the amino acid sequences set forth in SEQ ID NOS: 16 and 17, respectively.

[0277] A cDNA coding a ghrelin precursor derived from eel, *Xenopus Laevis* or rainbow trout was cloned from various cDNA libraries in the method described in Example 5 by plaque hybridization where the PCR-amplified DNA fragment described in Example 5 was used as a probe. The nucleotide sequence of the resulting cDNA clone was determined and confirmed to code the ghrelin precursor.

[0278] The resulting cDNA for the eel ghrelin precursor was composed of the nucleotide set forth in SEQ ID NO: 36, the cDNA for the frog ghrelin precursor was composed of the nucleotide set forth in SEQ ID NO: 37, and the cDNA for the rainbow trout ghrelin precursor was composed of the nucleotide set forth in SEQ ID NO: 38 or 39.

[0279] From rainbow trout, the cDNA coding the ghrelin-23 precursor set forth in SEQ ID NO: 38 and the cDNA coding the ghrelin-20 precursor set forth in SEQ ID NO: 39 were obtained.

[0280] From the nucleotide sequences of the above cDNAs, it was found that the eel ghrelin precursor has the amino acid sequence set forth in SEQ ID NO: 32, the frog ghrelin precursor has the amino acid sequence set forth in SEQ ID NO: 33, and the rainbow trout ghrelin precursor has the amino acid sequence set forth in SEQ ID NO: 34 or 35.

[0281] From rainbow trout, the amino acid sequence of ghrelin-23 precursor set forth in SEQ ID NO: 34 and the amino acid sequence of ghrelin-20 precursor in SEQ ID NO: 35 were found.

[0282] A cDNA for a bovine ghrelin precursor was cloned by the PCR method. That is, PCR was carried out where synthetic DNA having nucleotide sequences designed on the basis of amino acid sequences conserved among the rat, human and porcine-derived ghrelins and ghrelins-27 were used as primer and a bovine stomach cDNA library as template. The DNA fragment thus amplified had the nucleotide sequence set forth in SEQ ID NO: 24, coding a part of bovine ghrelin-27 precursor set forth in SEQ ID NO: 23. Accordingly, the bovine ghrelin-27 has the amino acid sequence set forth in SEQ ID NO: 22. In the DNA fragment amplified by the above PCR using the bovine stomach cDNA library as template, there was no DNA coding a ghrelin (28 amino acids) precursor.

[0283] The amino acids of the rat-, human- and porcine-derived ghrelins and the rat-, human-, porcine- and bovine-derived ghrelins-27 were very similar, and in particular, the amino acid sequences of 1st to 10th amino acids in the 7 ghrelins described above were completely identical with one another.

#### Example 9. Comparison of activity among various ghrelin derivatives

[0284] Peptide fragments obtained by partially digested the rat- and human-derived ghrelins by various proteases, or chemically synthesized peptides, were examined for their Ca releasing activity in order to determine a core amino acid sequence and the optimum chain length of a modifying fatty acid necessary for Ca-releasing activity. The Ca-releasing activity of ghrelin was expressed in terms of ghrelin concentration ( $EC_{50}$ , nM) at which 50 % of the maximum activity is achieved. Accordingly, lower  $EC_{50}$  values are indicative of higher activity.

Table 3

Comparison of the activity among various ghrelin derivatives					
Origin	SEQ ID NO.	Amino acids	Fatty acid	Ca-releasing	Remark ( $EC_{50}$ , nM)
			modification	activity	
human	3	1-28	Acyl (C : 8)	2.6	natural ghrelin
human	3	1-15	Acyl (C : 8)	7.0	
human	3	1-11	Acyl (C : 8)	15	
rat	2	1-28	Acyl (C : 8)	2.9	natural ghrelin
rat	2	1-15	Acyl (C : 8)	8.6	
rat	2	1-11	Acyl (C : 8)	15	
rat	2	1-10	Acyl (C : 8)	19	
rat	2	1-9	Acyl (C : 8)	38	
rat	2	1-8	Acyl (C : 8)	100	
rat	2	1-4	Acyl (C : 8)	480	
rat	2	16-28	Acyl (C : 8)	>10000	
rat	2	(1-28)+ (14-28)	Acyl (C : 8)	2.8	ghrelin-27
rat	2	1-28	Acyl (C : 16)	3.1	
rat	2	1-28	Acyl (C : 10)	2.6	



Table 3 (continued)

Comparison of the activity among various ghrelin derivatives					
Origin	SEQ ID NO.	Amino acids	Fatty acid	Ca-releasing	Remark
			modification	activity	(EC <sub>50</sub> , nM)
rat	2	1-28	Acyl (C : 6)	16	
rat	2	1-28	Acyl (C : 4)	280	
rat	2	1-28	Acyl (C : 2)	780	

[0285] The Ca-releasing activity of ghrelin is present in the side of the amino-terminal. A peptide of from the amino-terminal to 4th amino acid has a sufficient Ca-releasing activity, and a peptide of from the amino-terminal to 10th amino acid shows a strong Ca-releasing activity near to that of natural ghrelin. When the chain length of modifying fatty acid is C : 2 (acetyl group), a sufficient activity is brought about, and when the chain length is C : 8 (octanoyl group), the maximum Ca-releasing activity is achieved, and even if the number of carbon atoms of fatty acid is further increased to C : 10 (decanoyl group) or to C : 16, the strong Ca-releasing activity does not change. That is, when the fatty acid for modifying 3rd serine from the amino-terminal contains 8 or more carbon atoms, the strongest Ca-releasing activity is brought about.

#### Example 10. Synthesis of various ghrelin derivatives

##### (1) Synthesis of peptide derivatives

[0286] Amino acid derivatives other than Fmoc-D<sup>D</sup>Ser (C<sub>8</sub>H<sub>17</sub>) and Fmoc-Ser (C<sub>8</sub>H<sub>17</sub>), and synthesis reagents, were purchased from Perkin Elmer, Novabiochem or Watanabe Kagaku Co., Ltd. Peptide chain extension was performed by mainly using Applied Biosystem 433A synthesizer produced by Perkin Elmer, and a protected peptide derivative-resin was constructed by the Boc or Fmoc method. The protected peptide resin obtained by the Boc method was deprotected with anhydrous hydrogen fluoride (HF) in the presence of p-cresol thereby releasing the peptide, which was then purified. The protected peptide resin obtained by the Fmoc method was deprotected with trifluoroacetic acid (TFA) or dilute TFA containing various scavengers, and the released peptide was purified. Purification was performed in reversed phase HPLC on a C4 or C18 column. The purity of the purified product was confirmed by reverse phase HPLC, and its structure was confirmed by amino acid composition analysis and mass spectrometry.

[0287] The peptide of the present invention is produced by a conventional peptide synthesis method. For example, it can be produced by a method described, in chapter 2 and 3 of "Biochemical Experimental Course 1, Protein Chemistry IV" (Tokyo Kagaku Dojin) or in "Development of Medicines, a second series, Vol. 14, Peptide Synthesis (Hirokawa Shoten Co., Ltd). Accordingly, typical examples of the peptide of the present invention are shown below. Specifically, synthesis of acylated or alkylated peptides is exemplified below. Further, human-derived ghrelin (which may be abbreviated hereinafter to hGhrelin) or rat-derived ghrelin (which may be abbreviated hereinafter to rGhrelin) was reacted with trypsin or chymotrypsin or both the enzymes successively to give the following ghrelin fragments: 19. Ghrelin (16-28), 20. hGhrelin (1-15), 21. rGhrelin (1-15), 23. hGhrelin (1-11), 24. rGhrelin (1-11), 25. Ghrelin (1-10), 26. Ghrelin (1-9), 27. Ghrelin (1-8), and 30. Ghrelin (1-4). Then, these fragments were isolated by analytical HPLC and measured for their activity. 41. [N-Acetyl]-Ghrelin (1-10) was prepared in a usual manner by treating Ghrelin (1-10) with N-acetylsuccinimide. Compound No. 2 (rat ghrelin) made use of a natural material, and 10. [Ser<sup>3</sup>(Butyryl)]-rGhrelin, 11. [Ser<sup>3</sup>(Hexanoyl)]-rGhrelin, 12. [Ser<sup>3</sup>(Decanoyl)]-rGhrelin, 13. [Ser<sup>3</sup>(Lauroyl)]-rGhrelin, and 14. [Ser<sup>3</sup>(Palmitoyl)]-rGhrelin were synthesized in the same manner as in synthesis of Compound 1 (hGhrelin) and then measured for their activity.

##### [Main Abbreviations]

[0288] HMP resin; 4-hydroxymethyl-phenoxyethyl resin  
 Fmoc amide resin; 4-(2', 4'-dimethoxyphenyl-Fmoc-aminomethyl) phenoxyacetamido-ethyl resin  
 PAM resin; phenylacetamidomethyl resin  
 HBTU; 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate  
 TBTU; 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate  
 HOBt; 1-hydroxybenzotriazole  
 DCC; dicyclohexylcarbodiimide  
 DIPCI; diisopropylcarbodiimide  
 TFA; trifluoroacetic acid

DIPEA; diisopropylethylamine  
 TIPS; triisopropylsilane  
 Fmoc; fluorenylmethoxycarbonyl  
 Boc; *t*-butoxycarbonyl  
 5 Trt; trityl  
 Bu<sup>t</sup>; *t*-butyl  
 Pmc; 2,2,5,7,8-pentamethylchroman-6-sulfonyl  
 Pri; propionyl  
 PhPri; phenylpropionyl  
 10 Bzl; benzyl  
 Bom; benzyloxymethyl  
 Tos; toluenesulfonyl  
 Cl-Z; 2-chloro-benzyloxycarbonyl  
 Pis; 2-phenylisopropyl  
 15 Mtt; 4-methyltrityl  
 DMF; *N,N*-dimethylformamide  
 NMP; *N*-methylpyrrolidone  
 DMAP; 4-dimethylaminopyridine  
 HOSu; *N*-hydroxysuccinimide  
 20 Adod; 2-aminododecanoic acid  
 Aib; 2-aminoisobutylic acid  
 Ape; 5-aminopentanoic acid  
 Cha; cyclohexylalanine  
 Dap; 2, 3-diaminopropionic acid  
 25 Nal; naphthylalanine  
 Nle; norleucine  
 [Protecting amino acids used in synthesis]  
 Fmoc method:

30 Boc-Gly, Fmoc-Gly, Fmoc-Ser (Bu<sup>t</sup>), Fmoc-Ser (Trt), Fmoc-Glu (OBu<sup>t</sup>), Fmoc-His (Boc), Fmoc-Gln (Trt), Fmoc-Arg (Pmc), Fmoc-Lys (Boc), Fmoc-Pro, Fmoc-Leu, Fmoc-Ala, Fmoc-Val, Fmoc-Phe, Fmoc-DPhe, Fmoc-Ser (*n*-C<sub>8</sub>H<sub>17</sub>), Fmoc-D<sup>+</sup>Ser (*n*-C<sub>8</sub>H<sub>17</sub>), Fmoc-Cys (*n*-C<sub>8</sub>H<sub>17</sub>), Fmoc-Asp (OPis), Fmoc-Ser (Bzl), Fmoc-Cys (Trt), Fmoc-Dap (Octanoyl), Fmoc-2-L<sup>+</sup>Nal, Fmoc-2-D<sup>+</sup>Nal, Fmoc-Nle, Fmoc-Lys (Mtt), Fmoc-Aib-OH, Fmoc-Asp (O-C<sub>7</sub>H<sub>15</sub>)

35 Boc method:

Boc-Gly, Boc-Ser (Bzl), Boc-Ser (Ac), Boc-Ser (Pri), Boc-Glu (OBzl), Boc-His (Bom), Boc-Gln, Boc-Arg (Tos), Boc-Lys (Cl-Z), Boc-Pro, Boc-Leu, Boc-Ala, Boc-Val, Boc-Phe, Boc-Cys (*n*-C<sub>8</sub>H<sub>17</sub>), Boc-Ape Boc-Ser (*n*-C<sub>8</sub>H<sub>17</sub>)

40 [Units used]

(a) Analytical HPLC system

[0289] Unit: Shimadzu LC-10A System

45 Column: YMC PROTEIN-RP (4.6 mmφ×150 mm)

Column temperature: 40 °C

Eluent: A linear gradient of from 0 to 50 % acetonitrile for 20 minutes in 0.1% trifluoroacetic acid

Flow rate: 1 mL/min.

Detection: UV (210 nm)

50 Injection volume: 10 to 100 μl

(b) Preparative HPLC system

[0290] Unit: Waters 600 Multisolvant Delivery System

55 Columns:

YMC-Pack-ODS-A (5 μm, 20 mm×250 mm)

YMC-Pack-PROTEIN-RP (5 μm, C4, 10 mm×250 mm)

YMC-Pack PROTEIN-RP (5  $\mu$ m, C4, 20 mm $\times$ 250 mm)

YMC PROTEIN-RP (4.6 mm $\phi$  $\times$ 150 mm)

Eluent: A suitable linear gradient of acetonitrile concentration in 0.1 % trifluoroacetic acid

5 Flow rate: 10 mL/min. (for the column of an inner diameter of 20 mm), 3 mL/min. (for the column of an inner diameter of 10 mm), 1 mL/min. (for the column of an inner diameter of 4.6 mm) Detection: 210 nm, 260 nm

Injection: 10 to 2000  $\mu$ l (2000  $\mu$ l or more was injected via a pump)

(c) Mass spectrometer

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[0291] Unit: Finigan MAT TSQ700

Ion source: ESI

Detection ion mode: Positive

Spray voltage: 4.5 kV

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Capillary temperature: 250  $^{\circ}$ C

Mobile phase: A mixture of 0.2% acetic acid and methanol (1 : 1)

Flow rate: 0.2 mL/min.

Scan range: m/z 300 to 1,500

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(d) Analysis of amino acid sequence

[0292] Unit: Applied Biosystem 477A, 492 model sequencer manufactured by Perkin Elmer

(e) Analysis of amino acid composition

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[0293] Unit: L-8500 model amino acid analyzer manufactured by Hitachi, Co., Ltd.

[0294] Sample: Unless otherwise specified, the sample was hydrolyzed with 6 M HCl at 110  $^{\circ}$ C for 24 hours in a sealed tube.

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(2) Example of synthesis of a derivative having acyl serine or acyl threonine (Fmoc method, carboxyl-terminal amide derivatives)

Compound 1 hGhrelin: GSS(CO-C<sub>7</sub>H<sub>15</sub>)FLSPEHQRVQQRKESKKPPAKLQPR

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[0295] Fmoc-Arg(Pmc)-HMP-resin (403 mg, 0.25 mmol, ABI Co., Ltd) was treated with 20% piperazine for 20 minutes and subjected repeatedly to introduction of Fmoc-amino acid by HBTU/HOBt and elimination of Fmoc by piperazine sequentially to construct Fmoc-Ser(Bu<sup>t</sup>)-Ser(Trt)-Phe-Leu-Ser(tBu)-Pro-Glu(OBu<sup>t</sup>)-His(Boc)-Gln(Trt)-Arg(Pmc)-Val-Gln(Trt)-Gln(Trt)-Arg(Pmc)-Lys(Boc)-Glu(OBu<sup>t</sup>)-Ser(Bu<sup>t</sup>)-Lys(Boc)-Lys(Boc)-Pro-Pro-Ala-Lys(Boc)-Leu-Gln(Trt)-Pro-Arg(Pmc)-resin. After Boc-Gly was finally introduced by DCC/HOBt, the resulting protected peptide resin (1.3 g)

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was treated with 1 % TFA-5 % TIPS-methylene chloride solution (15 mL) for 30 minutes. The peptide resin was filtrated, washed several times with methylene chloride (30 mL), and washed with 5 % DIEA (10 mL) and then with methylene chloride (30 mL). The resulting de-Trt peptide resin (about 1.3 g) was swollen with NMP (10 mL), and octanoic acid (144.2 mg, 1.0 mmol) and DIPCI (126.2 mg, 1.0 mmol) were added thereto in the presence of DMAP (61.1 mg, 0.5 mmol) and allowed to react for 8 hours. The resin was recovered by filtration and washed with NMP and then with

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methylene chloride, followed by drying under vacuum to give about 1.2 g protected peptide resin wherein the side chain of 3rd serine was octanoylated. To this product was added a de-protecting reagent (10 mL) consisting of 88 % TFA-5 % phenol-2% TIPS-5 % H<sub>2</sub>O, and the mixture was stirred at room temperature for 2 hours. The resin was removed by filtration, and the filtrate was concentrated followed by adding ether to the resulting residues to form pre-

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cipitates. The precipitates were recovered by filtration and dried to give about 550 mg crude peptide. 200 mg of this product was dissolved in 10 mL water and applied to YMC-Pack PROTEIN-RP (C4, 20 mm $\times$ 250 mm) and eluted with a linear gradient (flow rate: 10 mL/min.) for 60 minutes of from 0 to 54 % acetonitrile in 0.1% trifluoroacetic acid. The desired fractions were collected and lyophilized to give about 120 mg of the desired product.

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(3) Example of synthesis of a derivative having acyl serine or acyl threonine (Fmoc method, carboxyl-terminal amide compounds)

Compound 3 Ghrelin (1-9)-NH<sub>2</sub>; GSS(CO-C<sub>7</sub>H<sub>15</sub>)FLSPEH-NH<sub>2</sub>

[0296] Fmoc-amide-resin (403 mg, 0.25 mmol, ABI Co., Ltd) was treated with 20% piperazine for 20 minutes and subjected repeatedly to introduction of Fmoc-amino acid by HBTU/HOBt and elimination of Fmoc by piperazine sequentially to construct Fmoc-Ser(Bu<sup>t</sup>)-Ser(Trt)-Phe-Leu-Ser(Bu<sup>t</sup>)-Pro-Glu(OBu<sup>t</sup>)-His(Boc)-resin. After Boc-Gly was finally introduced by DCC/HOBt, the resulting protected peptide resin (about 550 mg) was treated with 1 % TFA-5 % TIPS-methylene chloride solution (10 mL) for 30 minutes. The peptide resin was recovered by filtration, washed several times with methylene chloride (30 mL), and washed with 5 % DIEA (10 mL) and then with methylene chloride (30 mL). The resulting de-Trt peptide resin (about 750 mg) was swollen with NMP (10 mL), and octanoic acid (144.2 mg, 1.0 mmol) and DIPC1 (126.2 mg, 1 mmol) were added thereto in the presence of DMAP (61.1 mg, 0.5 mmol) and allowed to react for 4 hours. The resin was recovered by filtration and washed with NMP and then with methylene chloride, followed by drying under vacuum to give about 800 mg protected peptide resin wherein the side chain of 3rd serine was octanoylated. TFA (10 mL) was added to this product and stirred at room temperature for 30 minutes. The resin was removed by filtration, and the filtrate was concentrated followed by adding ether to the resulting residues to form precipitates. The precipitates were recovered by filtration and dried to give about 250 mg crude peptide. About 200 mg of this product was dissolved in 10 mL of 30 % aqueous acetic acid and applied to YMC-Pack PROTEIN-RP (C4, 20 mm×250 mm) and eluted with a linear gradient (flow rate: 10 mL/min.) for 60 minutes of from 0 to 54 % acetonitrile in 0.1% trifluoroacetic acid. The desired fractions were collected and lyophilized to give about 150 mg of the desired product.

(4) Example of synthesis of a derivative having acyl serine or acyl threonine (Boc method)

Compound 9 [Ser<sup>3</sup>(Propionyl)]-rGhrelin (1-28); GSS(CO-CH<sub>2</sub>CH<sub>3</sub>)FLSPEHQKAQQRKESKKPPAKLQPR

[0297] Protected rat ghrelin resin (4-28) was constructed from Boc-Arg (Tos)-Pam resin (0.75 g, 0.5 mmol) by Boc chemistry, and Boc-Ser (CO-CH<sub>2</sub>CH<sub>3</sub>)-OH, Boc-Ser (Bzl)-OH and Boc-Gly-OH were condensed with a half (1.4 g) of the resin. The resulting resin, 1.5 g, was treated with a mixture of HF and p-cresol (8.5 mL : 1.5 mL) at 0 °C for 1 hour, and the HF was evaporated. Ether was added to the residues, whereby 671 mg crude peptide was obtained. This sample was dissolved in 50% acetic acid (AcOH) and applied to a preparative column YMC-Pack-ODS-A (5 μm, 20 mm×250 mm) and eluted at a rate of 10 mL/min. by a gradient of from 0 to 95 % acetonitrile concentration in 0.1% TFA solution for 75 minutes. Those fractions containing the desired product were lyophilized to give 135.8 mg crude peptide. A part (0.5 mg) of this product was applied to YMC-A-302 column (C18, 4.6 mm×150 mm) and eluted at a flow rate of 1 mL/min. by a gradient of from 15 to 19% concentration acetonitrile. This purification procedure was repeated and the desired fractions were combined to give 0.41 mg of the desired product.

[0298] The following peptide derivatives having acyl serine or acyl threonine were produced in the same manner as in production of Compound 3 or 9 described above.

[0299] The results of the mass spectrometry and amino acid composition analysis of the peptide derivatives having acyl serine or acyl threonine are summarized below.

Compound 1. hGhrelin

ESI-MS 3371.0 (theoretical: 3370.9), amino acid composition: Ser; 3.53 (4), Glx; 5.91 (6), Gly; 1.02 (1), Ala; 1.00 (1), Val; 0.96 (1), Leu; 2, Phe; 1.06 (1), Lys; 3.90 (4), His; 0.97 (1), Arg; 2.87 (3), Pro; 3.87 (4)

Compound 3. Ghrelin (1-9)-amide

ESI-MS [M+H]; 1085.7 (theoretical: 1085.2), amino acid composition: Ser; 2.45 (3), Glx; 0.98 (1), Gly; 0.99 (1), Leu; 1, Phe; 0.99 (1), His; 1.08 (1), Pro; 0.97 (1)

Compound 4. [Ser<sup>2</sup>(Octanoyl), Ser<sup>3</sup>]-Ghrelin (1-9)-amide

ESI-MS [M+H]; 1085.8 (theoretical: 1085.2), amino acid composition: Ser; 2.46 (3), Glx; 0.98 (1), Gly; 0.99 (1), Leu; 1, Phe; 1.01 (1), His; 1.09 (1), Pro; 0.97 (1)

Compound 5. [Ser<sup>2</sup>(Octanoyl)]-Ghrelin (1-9)-amide

ESI-MS [M+H]; 1211.7 (theoretical: 1211.4), amino acid composition: Ser; 2.48 (3), Glx; 1.00 (1), Gly; 1.01 (1), Leu; 1, Phe; 1.00 (1), His; 1.11 (1), Pro; 0.98 (1)

Compound 8. [Ser<sup>3</sup>(Acetyl)]-rGhrelin

ESI-MS 3231.0 (theoretical: 3230.7), amino acid composition: Ser; 3.50 (4), Glx; 5.90 (6), Gly; 0.98 (1), Ala; 2.00 (2), Leu; 2, Phe; 1.01 (1), Lys; 4.97 (5), His; 0.99 (1), Arg; 1.99 (2), Pro; 3.99 (4)

Compound 9. [Ser<sup>3</sup>(Propionyl)]-rGhrelin

- ESI-MS 3245.0 (theoretical: 3242.8), amino acid composition: Ser; 3.42 (4), Glx; 5.93 (6), Gly; 1.00 (1), Ala; 2.00 (2), Leu; 2, Phe; 1.10 (1), Lys; 4.97 (5), His; 0.99 (1), Arg; 1.99 (2), Pro; 3.83 (4)
- Compound 15. [Ser<sup>3</sup>(3-Phenylpropionyl)]-hGhrelin  
ESI-MS 3377.0 (theoretical: 3376.9), amino acid composition: Ser; 3.06 (4), Glx; 5.92 (6), Gly; 0.93 (1), Ala; 0.98 (1), Val; 0.99 (1), Leu; 2, Phe; 1.13 (1), Lys; 4.03 (4), His; 1.08 (1), Arg; 3.00 (3), Pro; 3.76 (4)
- Compound 16. [Ser<sup>3</sup>(3-Octenoyl)]-hGhrelin  
ESI-MS 3369.0 (theoretical: 3368.9), amino acid composition: Ser; 3.59 (4), Glx; 5.91 (6), Gly; 1.00 (1), Ala; 1.02 (1), Val; 0.99 (1), Leu; 2, Phe; 1.15 (1), Lys; 3.97 (4), His; 0.98 (1), Arg; 2.93 (3), Pro; 3.88 (4)
- Compound 28. Ghrelin (1-8)-amide  
ESI-MS [M+H]<sup>+</sup> 948.5 (theoretical: 948.1), amino acid composition: Ser; 2.45 (3), Glx; 0.97 (1), Gly; 0.99 (1), Leu; 1, Phe; 1.00 (1), Pro; 0.97 (1)
- Compound 29. Ghrelin (1-7)-amide  
ESI-MS [M+H]<sup>+</sup> 819.6 (theoretical: 819.0), amino acid composition: Ser; 2.52 (3), Gly; 1.01 (1), Leu; 1, Phe; 1.02 (1), Pro; 1.09 (1)
- Compound 30. Ghrelin (1-6)-amide  
ESI-MS [M+H]<sup>+</sup> 722.4 (theoretical: 721.8), amino acid composition: Ser; 2.47 (3), Gly; 0.99 (1), Leu; 1, Phe; 1.00 (1)
- Compound 31. Ghrelin (1-5)  
ESI-MS [M+H]<sup>+</sup> 636.5 (theoretical: 635.8), amino acid composition: Ser; 1.78 (2), Gly; 0.99 (1), Leu; 1, Phe; 1.02 (1)
- Compound 32. Ghrelin (1-5)-amide  
ESI-MS [M+H]<sup>+</sup> 635.4 (theoretical: 634.8), amino acid composition: Ser; 1.67 (2), Gly; 1.01 (1), Leu; 1, Phe; 1.01 (1)
- Compound 33-2. Ghrelin (1-4)-amide  
ESI-MS [M+H]<sup>+</sup> 522.2 (theoretical: 521.6), amino acid composition: Ser; 1.65 (2), Gly; 0.99 (1), Phe; 1
- Compound 34. Ghrelin (1-3)-amide  
ESI-MS [M+H]<sup>+</sup> 375.2 (theoretical: 374.4), amino acid composition: Ser; 1.66 (2), Gly; 1
- Compound 35. [Lys<sup>8</sup>]-Ghrelin (1-8)-amide  
ESI-MS [M+H]<sup>+</sup> 947.9 (theoretical: 947.1), amino acid composition: Ser; 2.70 (3), Gly; 1.00 (1), Leu; 1, Phe; 1.00 (1), Lys; 0.99 (1), Pro; 1.00 (1)
- Compound 36. [Arg<sup>8</sup>]-Ghrelin (1-8)-amide  
ESI-MS [M+H]<sup>+</sup> 975.8 (theoretical: 975.2), amino acid composition: Ser; 2.70 (3), Gly; 1.00 (1), Leu; 1, Phe; 1.01 (1), Arg; 0.99 (1), Pro; 1.00 (1)
- Compound 37. [Lys<sup>6</sup>]-Ghrelin (1-6)-amide  
ESI-MS [M+H]<sup>+</sup> 763.6 (theoretical: 762.9), amino acid composition: Ser; 1.80 (2), Gly; 1.00 (1), Leu; 1, Phe; 1.01 (1), Lys; 1.00 (1)
- Compound 38. [Lys<sup>5</sup>]-Ghrelin (1-5)-amide  
ESI-MS [M+H]<sup>+</sup> 650.5 (theoretical: 649.8), amino acid composition: Ser; 1.79 (2), Gly; 0.99 (1), Phe; 1, Lys; 0.99 (1)
- Compound 39. [Phe<sup>4</sup>, Lys<sup>5</sup>]-Ghrelin (1-5)-amide  
ESI-MS [M+H]<sup>+</sup> 650.5 (theoretical: 649.8), amino acid composition: Ser; 1.79 (2), Gly; 0.99 (1), Phe; 1, Lys; 0.99 (1)
- Compound 40. [N-Aminopentanoyl]-Ghrelin (3-7)-amide  
ESI-MS [M+H]<sup>+</sup> 774.7 (theoretical: 774.0), amino acid composition: Ser; 1.80 (2), Leu; 1, Phe; 1.01 (1), Pro; 1.00 (1)
- Compound 43. [N-Glycyl]-Ghrelin (3-7)-amide  
ESI-MS [M+H]<sup>+</sup> 732.7 (theoretical: 731.9), amino acid composition: Ser; 1.80 (2), Gly; 1.00 (1), Leu; 1, Phe; 1.01 (1), Pro; 1.00 (1)
- Compound 44. [Leu<sup>2</sup>]-Ghrelin (1-7)-amide  
ESI-MS [M+H]<sup>+</sup> 845.7 (theoretical: 845.1), amino acid composition: Ser; 1.80 (2), Gly; 1.01 (1), Leu; 2, Phe; 1.02 (1), Pro; 0.99 (1)
- Compound 45. [His<sup>2</sup>]-Ghrelin (1-7)-amide  
ESI-MS [M+H]<sup>+</sup> 869.7 (theoretical: 869.0), amino acid composition after hydrolysis with propionic acid-hydrochloric acid (50/50) at 150 °C for 2 hours: Ser; 1.02 (2), Gly; 1.00 (1), Leu; 1, Phe; 1.00 (1), His; 0.95 (1), Pro; 0.99 (1)
- Compound 46. [Lys<sup>2</sup>]-Ghrelin (1-7)-amide  
ESI-MS [M+H]<sup>+</sup> 860.7 (theoretical: 860.1), amino acid composition after hydrolysis with propionic acid-hydrochloric acid (50/50) at 150 °C for 2 hours: Ser; 1.04 (2), Gly; 1.00 (1), Leu; 1, Phe; 1.00 (1), Lys; 1.00 (1), Pro; 1.00 (1)
- Compound 47. [Gly<sup>2</sup>]-Ghrelin (1-7)-amide  
ESI-MS [M+H]<sup>+</sup> 789.5 (theoretical: 788.9), amino acid composition after hydrolysis with propionic acid-hydrochloric acid (50/50) at 150 °C for 2 hours: Ser; 1.14 (2), Gly; 2.01 (2), Leu; 1, Phe; 1.00 (1), Pro; 1.00 (1)
- Compound 59. [Thr<sup>3</sup>(Octanoyl)]-hGhrelin  
ESI-MS M<sup>+</sup>; 3384.0 (theoretical: 3384.9), amino acid composition: Ala; 1.02 (1) Arg; 2.99 (3), Glx; 5.91 (6), Gly; 1.02 (1), His; 1.00 (1), Leu; 2 (2), Lys; 4.05 (4), Phe; 1.00 (1), Pro; 4.06 (4), Ser; 2.66 (3), Thr; 0.94 (1), Val; 0.96 (1)
- Compound 60. [Leu<sup>2</sup>, Thr<sup>3</sup>(Octanoyl)]-hGhrelin

ESI-MS M; 3410.0 (theoretical: 3411.0), amino acid composition: Ala; 1.01 (1), Arg; 2.95 (3), Glx; 5.92 (6), Gly; 1.01 (1), His; 1.01 (1), Leu; 3 (3), Lys; 4.02 (4), Phe; 1.01 (1), Pro; 4.00 (4), Ser; 1.81 (2), Thr; 0.96 (1), Val; 0.97 (1)  
Compound 69. [Ser<sup>3</sup>(4-Methylpentanoyl)]-hGhrelin

ESI-MS M; 3343.0 (theoretical: 3342.9), amino acid composition: Ala; 1.00 (1), Arg; 2.97 (3), Glx; 5.86 (6), Gly; 1.02 (1), His; 1.01 (1), Leu; 2, Lys; 4.00 (4), Phe; 1.01 (1), Pro; 3.99 (4), Ser; 3.54 (4), Val; 0.98 (1)

Compound 75. [Lys<sup>7</sup>]-Ghrelin (1-7)-amide

ESI-MS [M+H]; 850.5 (theoretical: 850.0), amino acid composition: Ser; 2.67 (3), Gly; 1.00 (1), Leu; 1, Phe; 1.00 (1), Lys; 1.00 (1)

#### (5) Example of synthesis of an amino-terminal acylated derivative

Compound 6. [N-Octanoyl, Ser<sup>3</sup>]-Ghrelin (1-9)-amide; C<sub>7</sub>H<sub>15</sub>CO-GSSFLSPEH-NH<sub>2</sub>

[0300] Fmoc-amide resin (403 mg, 0.25 mmol, ABI Co., Ltd) was treated with 20% piperazine for 20 minutes and subjected repeatedly to introduction of Fmoc-amino acid by HBTU/HOBt and elimination of Fmoc by piperazine sequentially to construct Fmoc-Gly-Ser(Bu<sup>t</sup>)-Ser(Bu<sup>t</sup>)-Phe-Leu-Ser(tBu)-Pro-Glu(OBu<sup>t</sup>)-His(Boc)-resin. After treatment with piperazine, the resulting peptide resin (550 mg) was washed with NMP, and DIPCl (126.2 mg, 1 mmol) and octanoic acid (144.2 mg, 1.0 mmol) were added thereto in the presence of HOBt (135.1 mg, 1 mmol) and allowed to react for 4 hours. The resin was recovered by filtration, washed with NMP and then with methylene chloride, and dried under vacuum to give about 600 mg protected peptide resin wherein the amino group in amino-terminal Gly was octanoylated. This product was deprotected with TFA (10 ml) (treatment for 30 minutes), to give 200 mg crude peptide. The whole of the sample was applied to YMC-Pack PROTEIN-RP (5 µm, C<sub>4</sub>, 20 mm×250 mm) and eluted with a linear gradient (flowrate: 10 mL/min) for 60 minutes of from 0 to 54 % acetonitrile in 0.1% trifluoroacetic acid. About 180 mg of the desired product was obtained. Measured values: ESI-MS [M+H]; 1085.6 (theoretical: 1085.2), amino acid composition: Ser; 2.47 (3), Glx; 0.98 (1), Gly; 1.00 (1), Leu; 1, Phe; 1.02 (1), His; 1.09 (1), Pro; 0.96 (1)

#### (6) Example of synthesis of a derivative containing serine having an alkyl side-chain

Compound 50. [Ser<sup>3</sup>(Octyl)]-Ghrelin (1-7)-amide; GSS(C<sub>8</sub>H<sub>17</sub>)FLSP-NH<sub>2</sub>

Fmoc-Ser (C<sub>8</sub>H<sub>17</sub>)

[0301] Under cooling on ice, sodium hydride (3.19g, 133 mmol) was added to a solution of Boc-Ser (12.3 g, 53.9 mmol) in DMF (300 ml) and stirred at room temperature for 1.5 hours. Octane iodide (11.0 ml, 60.9 mmol) was added thereto and stirred at room temperature for 16 hours. After water (40 ml) was added dropwise to the reaction solution under cooling on ice, the solvent was evaporated under vacuum. The resulting residues were purified by applying them to silica gel column chromatography (gel; Art9385, Merck Co., Ltd, eluent; dichloromethane : methanol : acetic acid = 120 : 10 : 1), to give 6.88 g Boc-Ser (C<sub>8</sub>H<sub>17</sub>) (yield, 36.2%) as pale yellow oily matter. Trifluoroacetic acid (120 ml) was added to this product, Boc-Ser (C<sub>8</sub>H<sub>17</sub>) (6.88 g, 21.7 mmol), under cooling on ice and stirred for 0.5 hour at room temperature. After the trifluoroacetic acid was evaporated, the resulting residues were dissolved in diethyl ether (120 ml), and 4N HCl-dioxane (22 ml) was added thereto and stirred for 1 hour under cooling on ice. The precipitated crystals were recovered by filtration to give 5.23 g H-Ser (C<sub>8</sub>H<sub>17</sub>)-HCl (yield, 96.3 %) as colorless crystals. After triethylamine (1.40 ml, 10 mmol) was added to a suspension (50 ml) of this product H-Ser (C<sub>8</sub>H<sub>17</sub>)-HCl (2.54 g, 10.0 mmol) in 10 % sodium hydrogen carbonate, a solution of Fmoc-Osu (5.00 g, 14.8 mmol) in 1,2-dimethoxyethane (20 ml) was added dropwise thereto over the period of 10 minutes and stirred at room temperature for 16 hours. The insolubles were filtered off, then dichloromethane was added to the filtrate, and the organic phase was separated and washed with 13 % NaCl solution. The organic phase was dried over anhydrous sodium sulfate, and the solvent was evaporated. The resulting residues were purified by applying them to silica gel column chromatography (gel; BW-300, Fuji Silicia Co., Ltd, eluent; dichloromethane : methanol = 93 : 7), to give 2.75 g Fmoc-Ser (C<sub>8</sub>H<sub>17</sub>) (yield: 62.6 %) as colorless crystals. R<sub>f</sub> = 0.45 (CHCl<sub>3</sub> : MeOH = 9 : 1, Silica gel 60F<sub>254</sub>, MERCK Co., Ltd). Fmoc-<sup>D</sup>Ser(C<sub>8</sub>H<sub>17</sub>) : R<sub>f</sub> = 0.45 (CHCl<sub>3</sub> : MeOH = 9 : 1, Silica gel 60F<sub>254</sub>, MERCK Co., Ltd).

[0302] Fmoc-amide resin (400 mg, 0.25 mmol, ABI Co., Ltd) was treated with 20% piperazine for 20 minutes and subjected repeatedly to introduction of Fmoc-amino acid by HBTU/HOBt and elimination of Fmoc by piperazine sequentially to construct Fmoc-Ser(Bu<sup>t</sup>)-Ser(C<sub>8</sub>H<sub>17</sub>)-Phe-Leu-Ser(Bu<sup>t</sup>)-Pro-resin. After Boc-Gly was finally introduced by DCC/HOBt, a part (250 mg) of the resulting protected peptide resin was treated with TFA (10 mL) for 30 minutes. The resin was removed by filtration, and the filtrate was concentrated followed by adding ether to the resulting residues to give about 120 mg crude peptide as precipitates. This product was dissolved in 5 % AcOH (10 mL) and applied to YMC-Pack-ODS-A (5 µm, 20 mm×250 mm) and eluted with a linear gradient (flow rate: 10 mL/min.) for 60 minutes of

from 0 to 60 % acetonitrile in 0.1% trifluoroacetic acid. The desired fractions were collected and lyophilized to give about 40 mg of the desired product.

Compound 84. [N-Aminopentanoyl, Ser<sup>3</sup>(Octyl)]-Ghrelin (3-5)-benzyl amide;

5 H-Ape-Ser(C<sub>8</sub>H<sub>17</sub>)-Phe-Leu-NH-CH<sub>2</sub>-Ph

[0303] Oxime resin (230 mg/0.25 mmol, Novabiochem Co., Ltd) was placed in a reaction vessel equipped with a glass filter, and Boc-Leu-OH · H<sub>2</sub>O (190 mg, 0.75 mmol) previously dissolved in methylene chloride (DCM) and dried over MgSO<sub>4</sub>, DCC (160 mg, 0.75 mmol), and 5 ml DCM were added thereto and shaken overnight. The reaction product was washed several times with a suitable amount of DCM, DCM/EtOH (1 : 1) and DCM in this order. After introduction of Leu, <1> 10 ml of 25% TFA/DCM was added thereto and shaken for 30 minutes, and the resin was washed several times with DCM, isopropyl alcohol (iPrOH), DCM and DMF in this order, and <2> a solution prepared by dissolving 0.75 mmol (3 equivalents) of Boc-amino acid, 0.75 mmol (3 equivalents) of TBTU and 0.75 mmol (3 equivalents) of HOBT and then adding 1.25 mmol (5 equivalents) of DIPEA thereto in 5 mL DMF in an Erlenmeyer flask was introduced into the reaction vessel and shaken for 1 hour; this operation was repeatedly carried out to condense amino acids sequentially. Finally, Boc-NH-(CH<sub>2</sub>)<sub>4</sub>-CO-Ser(C<sub>8</sub>H<sub>17</sub>)-Phe-Leu-Oxime resin, 370 mg, was obtained. The resin was suspended in about 5 mL DMF, and benzylamine hydrochloride (180 mg, 1.25 mmol), triethylamine (173 µL, 1.25 mmol) and acetic acid (72 µL, 1.25 mmol) were added thereto, and the mixture was stirred. After 24 hours, the resin was filtered off, and the filtrate was evaporated, and the resulting Boc-protected peptide was precipitated in 10 mL of 1N HCl. This product was washed with water and dried, and 5 mL TFA was added thereto and reacted for 30 minutes thereby eliminate the Boc. The TFA was evaporated, and the product was precipitated with ether (Et<sub>2</sub>O), whereby the desired product [N-Aminopentanoyl, Ser<sup>3</sup>(Octyl)]-Ghrelin (3-5)-benzylamide, 110 mg, was obtained. Compounds 82, 83 and 85 were synthesized in the same manner.

[0304] The following peptide derivatives having alkyl serine, except for Compounds 82 to 85, were produced in the same manner as in production of Compound 50 described above.

25 [0305] The results of the mass spectrometry and amino acid composition analysis of the peptide derivatives having alkyl serine are summarized below.

Compound 17. [Ser<sup>3</sup>(Octyl)]-hGhrelin

30 ESI-MS; 3357.0 (theoretical: 3356.9), amino acid composition: Ser; 2.92 (3+1), Glx; 5.94 (6), Gly; 1.00 (1), Ala; 0.98 (1), Val; 0.99 (1), Leu; 2, Phe; 1.13 (1), Lys; 4.04 (4), His; 1.09 (1), Arg; 3.01 (3), Pro; 3.89 (4)

Compound 50. [Ser<sup>3</sup>(Octyl)]-Ghrelin (1-7)-amide

ESI-MS [M+H]; 805.5 (theoretical: 805.0), amino acid composition after hydrolysis with propionic acid-hydrochloric acid (50/50) at 150 °C for 2 hours: Ser; 0.86 (2+1), Gly; 1.01 (1), Leu; 1, Phe; 1.06 (1), Pro; 0.95 (1)

Compound 51. [Ser<sup>3</sup>(Octyl), <sup>D</sup>Phe<sup>4</sup>]-Ghrelin (1-7)-amide

35 ESI-MS [M+H]; 805.4 (theoretical: 805.0), amino acid composition after hydrolysis with propionic acid-hydrochloric acid (50/50) at 150 °C for 2 hours: Ser; 0.97 (2+1), Gly; 1.00 (1), Leu; 1, Phe; 1.05 (1), Pro; 1.16 (1)

Compound 52. [<sup>D</sup>Ser<sup>3</sup>(Octyl)]-Ghrelin (1-7)-amide

ESI-MS [M+H]; 805.4 (theoretical: 805.0), amino acid composition after hydrolysis with propionic acid-hydrochloric acid (50/50) at 150 °C for 2 hours: Ser; 1.51 (2+1), Gly; 1.00 (1), Leu; 1, Phe; 1.00 (1), Pro; 1.00 (1)

40 Compound 53. [<sup>D</sup>Ser<sup>3</sup>(Octyl), <sup>D</sup>Phe<sup>4</sup>]-Ghrelin (1-7)-amide

ESI-MS [M+H]; 805.5 (theoretical: 805.0), amino acid composition after hydrolysis with propionic acid-hydrochloric acid (50/50) at 150 °C for 2 hours: Ser; 1.51 (2+1), Gly; 1.00 (1), Leu; 1, Phe; 1.00 (1), Pro; 1.01 (1)

Compound 67. [Ser<sup>3</sup>(Bzl)]-hGhrelin

45 ESI-MS M; 3335.0 (theoretical: 3334.8), amino acid composition: Ala; 1.00 (1), Arg; 2.96 (3), Glx; 5.92 (6), Gly; 1.00 (1), His; 1.01 (1), Leu; 2 (2), Lys; 4.00 (4), Phe; 1.02 (1), Pro; 4.08 (4), Ser; 3.58 (4), Val; 0.98 (1)

Compound 76. [N-Aminopentanoyl, Ser<sup>3</sup>(Octyl), Lys<sup>5</sup>]-Ghrelin (3-5)-amide

ESI-MS [M+H]; 591.5 (theoretical: 590.8), amino acid composition: Ser; 0.45 (1), Phe; 1, Lys; 1.00 (1)

Compound 77. [N-Aminopentanoyl, <sup>D</sup>Ser<sup>3</sup>(Octyl), <sup>D</sup>Phe<sup>4</sup>, Lys<sup>5</sup>]-Ghrelin (3-5)-amide

ESI-MS [M+H]; 591.5 (theoretical: 590.8), amino acid composition: Ser; 0.45 (1), Phe; 1, Lys; 1.01 (1)

50 Compound 78. [Aib<sup>1</sup>, His<sup>2</sup>, Ser<sup>3</sup>(Octyl), Lys<sup>5</sup>]-Ghrelin (1-5)-amide

ESI-MS [M+H]; 714.6 (theoretical: 713.9), amino acid composition: Ser; 0.45 (1), Phe; 1, His; 1.01 (1), Lys; 1.00 (1)

Compound 79. [Aib<sup>1</sup>, His<sup>2</sup>, <sup>D</sup>Ser<sup>3</sup>(Octyl), <sup>D</sup>Phe<sup>4</sup>, Lys<sup>5</sup>]-Ghrelin (1-5)-amide

ESI-MS [M+H]; 714.5 (theoretical: 713.9), amino acid composition: Ser; 0.44 (1), Phe; 1, His; 1.00 (1), Lys; 1.01 (1)

Compound 81. [N-Aminopentanoyl, Ser<sup>3</sup>(Octyl)]-Ghrelin (3-5)-amide

55 ESI-MS [M+H]; 576.5 (theoretical: 575.8), amino acid composition: Ser; 0.49 (1), Leu; 1, Phe; 0.99 (1)

Compound 82. [N-Aminopentanoyl, Ser<sup>3</sup>(Octyl)]-Ghrelin (3-5)-methanamide

ESI-MS [M+H]; 590.6 (theoretical: 589.8), amino acid composition: Ser; 0.49 (1), Leu; 1, Phe; 0.99 (1)

Compound 83. [N-Aminopentanoyl, Ser<sup>3</sup>(Octyl)]-Ghrelin (3-5)-ethylamide

ESI-MS [M+H]<sup>+</sup>; 604.3 (theoretical: 603.8), amino acid composition: Ser; 0.50 (1), Leu; 1, Phe; 0.99 (1)

Compound 84. [N-Aminopentanoyl, Ser<sup>3</sup>(Octyl)]-Ghrelin (3-5)-benzylamide

ESI-MS [M+H]<sup>+</sup>; 666.5 (theoretical: 665.9), amino acid composition: Ser; 0.46 (1), Leu; 1, Phe; 0.98 (1)

Compound 85. [N-Aminopentanoyl, Ser<sup>3</sup>(Octyl)]-Ghrelin (3-5)-aminoethylamide

ESI-MS [M+H]<sup>+</sup>; 619.6 (theoretical: 618.9), amino acid composition: Ser; 0.47 (1), Leu; 1, Phe; 0.99 (1)

(7) Example of synthesis of a derivative containing cysteine having an alkyl side-chain

Compound 48. [Cys<sup>3</sup>(Octyl)]-Ghrelin (1-7)-NH<sub>2</sub>; GSC(C<sub>8</sub>H<sub>17</sub>)FLSP-NH<sub>2</sub>

[0306] Fmoc-amide-resin (403 mg, 0.25 mmol, ABI Co., Ltd) was treated with 20% piperazine for 20 minutes and subjected repeatedly to introduction of Fmoc-amino acid by HBTU/HOBt and elimination of Fmoc by piperazine sequentially to construct Fmoc-Ser(Bu<sup>t</sup>)-Cys(C<sub>8</sub>H<sub>17</sub>)-Phe-Leu-Ser(Bu<sup>t</sup>)-Pro resin. After Boc-Gly was finally introduced by DCC/HOBt, the resulting protected peptide resin (550 mg) was treated with TFA (10 mL) for 30 minutes. The resin was removed by filtration, and the filtrate was concentrated followed by adding ether to the resulting residues to give about 120 mg crude peptide as precipitates. This product was dissolved in 10 mL of 5 % acetic acid and applied to YMC-Pack-ODS-A (5 µm, 20 mm×250 mm) and eluted with a linear gradient (flow rate: 10 mL/min.) for 60 minutes of from 0 to 60 % acetonitrile in 0.1% trifluoroacetic acid. The desired fractions were collected and lyophilized to give about 44 mg of the desired product.

Compound 68. [Cys<sup>3</sup>(Trt)]-hGhrelin;

GSC(C-Ph<sub>3</sub>)FLSPEHQVRVQQRKESKKPPAKLQPR

[0307] Fmoc-Arg(Pmc)-resin (403 mg, 0.25 mmol, ABI Co., Ltd) was treated with 20% piperazine for 20 minutes and subjected repeatedly to introduction of Fmoc-amino acid by HBTU/HOBt and elimination of Fmoc by piperazine sequentially to construct Fmoc-Ser(Bu<sup>t</sup>)-Cys(Trt)-Phe-Leu-Ser(tBu)-Pro-Glu(OBu<sup>t</sup>)-His(Boc)-Gln(Trt)-Arg(Pmc)-Val-Gln(Trt)-Gln(Trt)-Arg(Pmc)-Lys(Boc)-Glu(OBu<sup>t</sup>)-Ser(Bu<sup>t</sup>)-Lys(Boc)-Lys(Boc)-Pro-Pro-Ala-Lys(Boc)-Leu-Gln(Trt)-Pro-Arg(Pmc)-HMP resin. After Boc-Gly was finally introduced by DCC/HOBt, the resulting protected peptide resin (1.4 g) was recovered. TFA (15 mL) was added to a part (400 mg) of the resulting resin and stirred at room temperature for 1 hour. The resin was removed by filtration, and the filtrate was concentrated followed by adding ether to the resulting residues to form precipitates. About 90 mg of the precipitates were dissolved in 40 mL water, then applied to YMC-Pack PROTEIN-RP (C4, 20 mm×250 mm) and eluted with a linear gradient (flow rate: 10 mL/min.) for 60 minutes of from 0 to 54 % acetonitrile in 0.1% trifluoroacetic acid. The desired fractions were collected and lyophilized to give about 60 mg of the desired product.

[0308] The following peptide derivatives having alkyl cysteine were produced in the same manner as in production of Compound 48 or 68 described above.

[0309] The results of the mass spectrometry and amino acid composition analysis of the peptide derivatives having alkyl cysteine are summarized below.

Compound 18. [Cys<sup>3</sup>(Octyl)]-rGhrelin

ESI-MS; 3317.0 (theoretical: 3316.9), amino acid composition: Ser; 2.69 (3), Glx; 5.90 (6), Gly; 1.00 (1), Ala; 1.99 (2), Leu; 2, Phe; 1.02 (1), Lys; 4.97 (5), His; 0.99 (1), Arg; 1.98 (2), Pro; 3.87 (4)

Compound 48. [Cys<sup>3</sup>(Octyl)]-Ghrelin (1-7)-amide

ESI-MS [M+H]<sup>+</sup>; 821.7 (theoretical: 821.1), amino acid composition after hydrolysis with propionic acid-hydrochloric acid (50/50) at 150 °C for 2 hours: Ser; 0.60 (2), Gly; 1.08 (1), Leu; 1, Phe; 1.06 (1), Pro; 0.96 (1)

Compound 49. [Cys<sup>3</sup>(Octyl), <sup>D</sup>Phe<sup>4</sup>]-Ghrelin (1-7)-amide

BSI-MS[M+H]<sup>+</sup>; 821.6 (theoretical: 821.1), amino acid composition after hydrolysis with propionic acid-hydrochloric acid (50/50) at 150 °C for 2 hours: Ser; 0.58 (2), Gly; 1.02 (1), Leu; 1, Phe; 1.06 (1), Pro; 0.97 (1)

Compound 68. [Cys<sup>3</sup>(Trt)]-hGhrelin

ESI-MS 3503.0 (theoretical: 3503.1), amino acid composition: Ser; 2.42 (3), Glx; 5.77 (6), Gly; 1.00 (1), Ala; 1.01 (1), Val; 0.94 (1), Leu; 2, Phe; 0.99 (1), Lys; 3.94 (4), His; 0.99 (1), Arg; 2.92 (3), Pro; 3.81 (4)



## (8) Example of synthesis of a peptide derivative containing N-methyl amino acids

Compound 86. [N-Aminopentanoyl, Ser<sup>3</sup>(Octyl), MePhe<sup>4</sup>, MeLeu<sup>5</sup>]-Ghrelin (3-5)-amide; NH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-CO-Ser(C<sub>8</sub>H<sub>17</sub>)-MePhe-MeLeu-NH<sub>2</sub>

[0310] Fmoc-amide resin (0.40 g, 0.25 mmol) was placed in a reaction vessel equipped with a glass filter, and 15 mL of 20% piperidine in NMP was added thereto and shaken for 20 minutes, thus removing the Fmoc group. Thereafter, 15 mL NMP, 1.0 mmol (4 equivalents) of Fmoc-MeLeu-OH, 1.0 mmol (4 equivalents) of TBTU, 1.0 mmol (4 equivalents) of HOBt and 1.0 mmol (4 equivalents) of DIPEA were added thereto and shaken for 1 hour to condense the Fmoc-MeLeu. Thereafter, the peptide chain was extended by repeatedly carrying out removal of Fmoc group by 20 % piperidine and condensation of Fmoc-amino acid (3 equivalents) by bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (3 equivalents) in the presence of 2.25 mmol (9 equivalents) of DIPEA. The conclusion of the condensation reaction was confirmed by deprotecting a small amount of the resin with TFA and examining it by HPLC and mass spectrometry (MS). After Boc-NH-(CH<sub>2</sub>)<sub>4</sub>-CO-Ser(O-C<sub>8</sub>H<sub>17</sub>)-MePhe-MeLeu-resin was obtained, this resin was treated with TFA for 30 minutes, whereby the resin was cleaved to de-protect the peptide. After the TFA was evaporated, the peptide was washed with ether (Et<sub>2</sub>O) to give 120 mg NH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-CO-Ser(C<sub>8</sub>H<sub>17</sub>)-MePhe-MeLeu-NH<sub>2</sub>. This product was applied to YMC-Pack ODS-A (C18, 20 mm×250 mm) and eluted with a linear gradient (flow rate: 10 mL/min.) for 60 minutes of 0 to 54 % acetonitrile in 0.1% trifluoroacetic acid. The desired fractions were collected and lyophilized to give 70 mg of the desired product. After this derivative was hydrolyzed with propionic acid-HCl (50/50) at 150 °C for 2 hours, the amount of the peptide was quantified using the ratio of the peak area of aminopentanoic acid detected in the amino acid analyzer to that of 10 nmol aminopentanoic acid as a standard.

ESI-MS [M+H]<sup>+</sup>; 604.5 (theoretical: 603.8), detected amino acids after hydrolysis with propionic acid-HCl (50/50) at 150 °C for 2 hours: Ser, Ape.

## (9) Synthesis of a mixed-disulfide derivative

Compound 57. [Cys<sup>3</sup>(S-Heptyl)]-hGhrelin;

GSC(S-C<sub>7</sub>H<sub>15</sub>)FLSPEHQRVQQRKESKKPPAKLQPR

[0311] A de-protecting reagent (15 mL) consisting of 88 % TFA-5 % phenol-2 % TIPS-5 % H<sub>2</sub>O was added to a protected peptide-HMP resin (1 g) obtained by synthesis in the same manner as in production of Compound 68, and then stirred at room temperature for 2 hours. The resin was removed by filtration, and the filtrate was concentrated followed by adding ether to the resulting residues, whereby about 550 mg crude [Cys<sup>3</sup>]-hGhrelin powder was obtained. This product was applied to YMC-Pack ODS-A (C18, 20 mm×250 mm) and eluted with a linear gradient (flow rate: 10 mL/min.) for 60 minutes of from 0 to 54 % acetonitrile in 0.1 % trifluoroacetic acid. The desired fractions were collected and lyophilized to give 300 mg [Cys<sup>3</sup>]-hGhrelin (1-28). 40 mg (11.4 μmol) of this product was dissolved in water (20 mL), and 1 mL solution of 4,4'-dithiodipyridine (7.5 mg, 34.2 μmol) in acetonitrile was added thereto and left for 1 hour. After the conclusion of the reaction was confirmed, the reaction solution was washed several times with chloroform to remove an excess of the 4,4'-dithiodipyridine and pyridone derivative. The aqueous layer (10 mL) containing [thiopyridyl Cys<sup>3</sup>]-hGhrelin (1-28) was adjusted to pH 7.4 with aq. 5 % NH<sub>3</sub>, and a solution of 1-heptane [sic.]thiol (4.5mg, 34.2 μmol) in 2 mL acetonitrile was added thereto. After 1 hour, the reaction solution was applied to YMC-Pack ODS-A (C18, 20 mm×250 mm) and eluted with a linear gradient (flow rate: 10 mL/min) for 60 minutes of from 0 to 54 % acetonitrile in 0.1% trifluoroacetic acid. The desired fractions were collected and lyophilized to give 15 mg of the desired product.

Compound 57. [Cys<sup>3</sup>(S-Heptyl)]-hGhrelin

ESI-MS 3391.0 (theoretical: 3391.0), amino acid composition: Ser; 2.76 (3), Glx; 5.81 (6), Gly; 0.99 (1), Ala; 1.01 (1), Val; 0.95 (1), Leu; 2, Phe; 0.99 (1), Lys; 3.95 (4), His; 0.99 (1), Arg; 2.93 (3), Pro; 3.84 (4)

## (10) Examples of synthesis of a derivative having an amide in a side chain at the 3rd position and an ester in the reverse direction

Compound 55. [Asp<sup>3</sup>(NH-Heptyl)]-hGhrelin;

GSD(NH-C<sub>7</sub>H<sub>15</sub>)FLSPEHQRVQQRKESKKPPAKLQPR

[0312] Fmoc-Arg(Pmc)-HMP-resin (403 mg, 0.25 mmol, ABI Co., Ltd) was treated with 20% piperazine for 20 minutes and subjected repeatedly to introduction of Fmoc-amino acid by HBTU/HOBt and elimination of Fmoc by piperazine

sequentially to construct Fmoc-Ser(Bu<sup>t</sup>)-Asp(OPis)-Phe-Leu-Ser(tBu)-Pro-Glu(OBu<sup>t</sup>)-His(Boc)-Gln(Trt)-Arg(Pmc)-Val-Gln(Trt)-Gln(Trt)-Arg(Pmc)-Lys(Boc)-Glu(OBu<sup>t</sup>)-Ser(Bu<sup>t</sup>)-Lys(Boc)-Lys(Boc)-Pro-Pro-Ala-Lys(Boc)-Leu-Gln(Trt)-Pro-Arg(Pmc)-HMP resin. After Boc-Gly was finally introduced by DCC/HOBt, the resulting protected peptide resin (1.3 g) was treated with 4 % TFA-methylene chloride solution (15 mL) for 15 minutes. The peptide resin was recovered by filtration and washed several times with methylene chloride (30 mL), washed with 4% DIEA (10 mL) and then with methylene chloride (30 mL).

[0313] The resulting de-Pis peptide resin (about 1.3 g) was swollen with NMP (10 mL), and water-soluble carbodiimide hydrochloride (191.7 mg, 1.0 mmol), HOBt (135.2 mg, 1.0 mmol) and n-heptylamine (115.2 mg, 1.0 mmol) were added thereto and allowed to react for 8 hours.

[0314] The resin was recovered by filtration, washed with NMP and methylene chloride, and dried under vacuum to give about 1.2 g protected peptide resin where the Asp 3 residue was heptylamidated. A deprotecting reagent (10 mL) consisting of 88 % TFA-5% phenol-2% TIPS-5% H<sub>2</sub>O was added thereto and stirred at room temperature for 2 hours. The resin was removed by filtration, and the filtrate was concentrated followed by adding ether to the resulting residues to form precipitates. The precipitates were recovered by filtration and dried to give about 550 mg crude peptide.

[0315] 200 mg of this product was dissolved in 10 mL water and applied to YMC-Pack PROTEIN-RP (C<sub>4</sub>, 20 mm×250 mm) and eluted with a linear gradient (flow rate: 10 mL/min.) for 60 minutes of from 0 to 54 % acetonitrile in 0.1 % trifluoroacetic acid. The desired fractions were collected and lyophilized to give about 120 mg of the desired product.

Compound 61. [Lys<sup>3</sup>(Octanoyl)]-hGhrelin;

GSK(CO-C<sub>7</sub>H<sub>15</sub>)FLSPEHQRVQQRKESKKPPAKLQPR

[0316] Fmoc-Arg(Pmc)-HMP-resin (403 mg, 0.25 mmol, a product of ABI Co., Ltd) was treated with 20% piperazine for 20 minutes and subjected repeatedly to introduction of Fmoc-amino acid by HBTU/HOBt and elimination of Fmoc by piperazine sequentially to construct Boc-Gly-Ser(tBu)-Lys(Mtt)-Phe-Leu-Ser(tBu)-Pro-Glu(OBu<sup>t</sup>)-His(Boc)-Gln(Trt)-Arg(Pmc)-Val-Gln(Trt)-Gln(Trt)-Arg(Pmc)-Lys(Boc)-Glu(OBu<sup>t</sup>)-Ser(Bu<sup>t</sup>)-Lys(Boc)-Lys(Boc)-Pro-Pro-Ala-Lys(Boc)-Leu-Gln(Trt)-Pro-Arg(Pmc)-HMP resin. About 300 mg of the resulting protected peptide resin was treated with 1 % TFA-5 % TIPS-methylene chloride solution (15 mL) for 60 minutes.

[0317] The peptide resin was recovered by filtration and washed several times with methylene chloride (30 mL), washed with 10 % DIEA (10 mL) and then with methylene chloride (30 mL). The resulting de-Mtt peptide resin (about 300 mg) was swollen with NMP (2 mL), and octanoic acid (40  $\mu$ L, 0.25 mmol) and DCC (52 mg, 0.25 mmol) were added thereto in the presence of HOBt (34 mg, 0.25 mmol) and allowed to react overnight.

[0318] The resin was recovered by filtration, washed with NMP and then with methylene chloride, and dried under vacuum to give about 300 mg protected peptide resin where the lysine 3rd residue was octanoylated. A deprotecting reagent (5 mL) consisting of 88 % TFA-5% phenol-2% TIPS-5% H<sub>2</sub>O was added thereto and stirred at room temperature for 2 hours. The resin was removed by filtration, and the filtrate was concentrated followed by adding ether to the resulting residues to form precipitates. The precipitates were separated by filtration and dried to give about 234 mg crude peptide.

[0319] This product was dissolved in 6 mL acetic acid and applied to YMC-Pack ODS-A (5  $\mu$ m, 20 mm×250 mm) and eluted with a linear gradient (flow rate: 10 mL/min.) for 60 minutes of from 0 to 60 % acetonitrile in 0.1 % trifluoroacetic acid. The desired fractions were collected and lyophilized to give about 100 mg powder. This product was dissolved in 2 mL of 50 % acetic acid and applied to YMC-Pack PROTEIN-RP (5  $\mu$ m, C<sub>4</sub>, 20 mm×250 mm) and eluted with a linear gradient (flow rate: 10 mL/min.) for 60 minutes of from 0 to 60 % acetonitrile in 0.1 % trifluoroacetic acid. The desired fractions were collected and lyophilized to give about 52 mg powder.

[0320] The following compounds were produced in the same manner as in preparation of Compound 55 or 61 described above.

[0321] The results of the mass spectrometry and amino acid composition analysis of the peptide derivatives synthesized by the conventional Fmoc method are summarized below.

Compound 54. [Asp<sup>3</sup>(O-Heptyl)]-hGhrelin (1-28)

ESI-MS 3371.0(theoretical: 3370.9), amino acid composition: Asx; 0.99 (1), Ser; 2.70 (3), Glx; 5.87 (6), Gly; 1.01 (1), Ala; 1.01 (1), Val; 0.94 (1), Leu; 2, Phe; 1.00 (1), Lys; 4.02 (4), His; 1.00 (1), Arg; 2.98 (3), Pro; 3.84 (4)

Compound 55. [Asp<sup>3</sup>(NH-Heptyl)]-hGhrelin (1-28)

ESI-MS 3370.0 (theoretical: 3369.9), amino acid composition: Asx; 0.88 (1), Ser; 2.95 (3), Glx; 5.97 (6), Gly; 1.21 (1), Ala; 1.03 (1), Val; 0.98 (1), Leu; 2, Phe; 1.00 (1), Lys; 3.94 (4), His; 0.92 (1), Arg; 2.91 (3), Pro; 3.99 (4)

Compound 56. [Dap<sup>3</sup>(Octanoyl)]-hGhrelin

ESI-MS M; 3370.0 (theoretical: 3369.9), amino acid composition: Ala; 1.02(1), Arg; 2.94 (3), Glx; 5.94 (6), Gly; 1.00 (1), His; 0.91 (1), Leu; 2 (2), Lys; 3.93 (4), Phe; 0.99 (1), Pro; 4.01 (4), Ser; 2.88 (3), Val; 0.98 (1), Dap; N.D.

Compound 58. [Adod<sup>3</sup>]-hGhrelin (1-28)

ESI-MS M; 3355.0 (theoretical: 3355.0), amino acid composition: Ala; 1.01 (1), Arg; 2.91 (3), Glx; 5.95 (6), Gly; 1.01 (1), His; 0.91 (1), Leu; 2 (2), Lys; 3.94 (4), Phe; 0.99 (1), Pro; 4.02 (4), Ser; 2.88 (3), Val; 0.96 (1)

Compound 61. [Lys<sup>3</sup>(Octanoyl)]-hGhrelin

ESI-MS M; 3412.0 (theoretical: 3412.0), amino acid composition: Ala; 1.05 (1), Arg; 3.05 (3), Glx; 6.02 (6), Gly; 1.00 (1), His; 1.00 (1), Leu; 2 (2), Lys; 5.11 (5), Phe; 0.97 (1), Pro; 4.20 (4), Ser; 2.68 (3), Val; 1.00 (1)

Compound 62. [Trp<sup>3</sup>]-hGhrelin

ESI-MS M; 3343.0 (theoretical: 3343.9), amino acid composition: Ala; 1.00 (1), Arg; 3.03 (3), Glx; 5.94 (6), Gly; 1.01 (1), His; 1.01 (1), Leu; 2 (2), Lys; 4.00 (4), Phe; 0.99 (1), Pro; 3.96 (4), Ser; 2.60 (3), Trp; N.D., Val; 0.98 (1)

Compound 63. [Phe<sup>3</sup>]-hGhrelin

ESI-MS M; 3305.0 (theoretical: 3304.8), amino acid composition: Ala; 0.99 (1), Arg; 2.96 (3), Glx; 5.86 (6), Gly; 1.00 (1), His; 1.00 (1), Leu; 2 (2), Lys; 3.98 (4), Phe; 2.01 (2), Pro; 3.99 (4), Ser; 2.67 (3), Val; 0.98 (1)

Compound 64. [Cha<sup>3</sup>]-hGhrelin

ESI-MS M; 3411.0 (theoretical: 3410.9), amino acid composition: Ala; 1.02 (1), Arg; 3.01 (3), Glx; 5.92 (6), Gly; 1.01 (1), His+Cha; 2.01 (1+1), Leu; 2 (2), Lys; 4.02 (4), Phe; 1.01 (1), Pro; 4.03 (4), Ser; 2.72 (3), Val; 0.97 (1)

Compound 65. [2-<sup>L</sup>NaI<sup>3</sup>]-hGhrelin

ESI-MS M; 3354.0 (theoretical: 3354.9), amino acid composition: Ala; 1.00 (1), Arg; 2.95 (3), Glx; 5.87 (6), Gly; 1.02 (1), His; 1.01 (1), Leu; 2 (2), Lys; 3.98 (4), Phe; 1.01 (1), Pro; 3.94 (4), Ser; 2.73 (3), Val; 0.97 (1), NaI; N.D. (1) Compound 66. [2-<sup>D</sup>NaI<sup>3</sup>]-hGhrelin

ESI-MS M; 3355.0 (theoretical: 3354.9), amino acid composition: Ala; 1.02 (1), Arg; 2.95 (3), Glx; 5.96 (6), Gly; 1.00 (1), His; 0.92 (1), Leu; 2 (2), Lys; 3.94 (4), Phe; 0.99 (1), Pro; 4.02 (4), Ser; 2.91 (3), Val; 0.98 (1), NaI; N.D. (2) Compound 70. [Leu<sup>3</sup>]-hGhrelin

ESI-MS M; 3270.0 (theoretical: 3270.8), amino acid composition: Ala; 0.99 (1), Arg; 2.95 (3), Glx; 5.88 (6), Gly; 1.01 (1), His; 1.00 (1), Leu; 3 (3), Lys; 3.96 (4), Phe; 1.00 (1), Pro; 3.89 (4), Ser; 2.65 (3), Val; 0.97 (1)

Compound 71. [Ile<sup>3</sup>]-hGhrelin

ESI-MS M; 3270.0 (theoretical: 3270.8), amino acid composition: Ala; 0.98 (1), Arg; 2.96 (3), Glx; 5.87 (6), Gly; 0.99 (1), His; 1.01 (1), Ile; 0.98 (1), Leu; 2 (2), Lys; 3.97 (4), Phe; 1.00 (1), Pro; 3.97 (4), Ser; 2.65 (3), Val; 0.98 (1) Compound 72. [Lys<sup>3</sup>(Octanoyl)]-hGhrelin

ESI-MS M; 3286.0 (theoretical: 3285.8), amino acid composition: Ala; 1.02 (1), Arg; 2.94 (3), Glx; 5.95 (6), Gly; 0.99 (1), His; 0.92 (1), Leu; 2 (2), Lys; 4.92 (5), Phe; 0.99 (1), Pro; 4.02 (4), Ser; 2.91 (4), Val; 0.99 (1)

Compound 73. [Nle<sup>3</sup>]-hGhrelin

ESI-MS M; 3270.0 (theoretical: 3270.8), amino acid composition: Ala; 1.01 (1), Arg; 2.98 (3), Glx; 5.92 (6), Gly; 1.02 (1), His; 1.01 (1), Leu; 2 (2), Lys; 4.01 (4), Phe; 1.01 (1), Pro; 4.01 (4), Ser; 2.71 (3), Val; 0.98 (1), Nle; N.D. (1) Compound 74. [Val<sup>3</sup>]-hGhrelin

ESI-MS M; 3256.0 (theoretical: 3256.8), amino acid composition: Ala; 0.98 (1), Arg; 2.96 (3), Glx; 5.84 (6), Gly; 1.00 (1), His; 1.01 (1), Leu; 2 (2), Lys; 3.97 (4), Phe; 0.99 (1), Pro; 3.94 (4), Ser; 2.64 (3), Val; 1.97 (2)

Compound 80. [Aib<sup>1</sup>, His<sup>2</sup>, <sup>D</sup>NaI<sup>3</sup>, <sup>D</sup>Phe<sup>4</sup>, Lys<sup>5</sup>]-Ghrelin (1-5)-amide; Ipamorelin

ESI-MS [M+H]; 712.5 (theoretical: 711.9), amino acid composition: Phe; 1, His; 1.00 (1), Lys; 1.00 (1)

## Example 11. Comparison of activity among ghrelin derivative peptide-type compounds

[0322] The Ca-releasing activities of the ghrelin derivative peptide-type compounds synthesized in Example 10 and the natural ghrelin peptide were measured in the same manner as in Example 1.

## (1) Modification of a side chain of 3rd serine

## A. Position of an octanoyl group

[0323] The significant structural feature of ghrelin lies in the octanoyl group on the hydroxyl group of 3rd serine. First, whether or not it is advantageous for exhibiting the activity that the position of serine to be octanoylated is the 3rd position was examined. In this examination, the intracellular Ca-releasing activity in CHO cells expressing rat GSH receptor was used as the indicator.

[0324] On the basis of ghrelin (1-9) amide (a short-chain ghrelin derivative) whose EC<sub>50</sub> value was kept at 5.4 nM, [serine<sup>2</sup> (octanoyl), serine<sup>3</sup>]-ghrelin (1-9) amide, [serine<sup>2</sup> (oatanoyl)]-ghrelin (1-9) amide, and [N<sup>α</sup>-octanoyl, serine<sup>3</sup>]-ghrelin (1-9) amide were synthesized, and their intracellular Ca-releasing activity was examined.

[0325] The results are summarized in Table 4.

Table 4

## Ghrelin derivative activity 1

Compound Structure	Ca-releasing activity EC <sub>50</sub> (nM)
1. human Ghrelin GSS(CO-C <sub>7</sub> H <sub>15</sub> )FLSPEHQRVQQRKESKKPPAKLQPR	1.3
2. rat Ghrelin GSS(CO-C <sub>7</sub> H <sub>15</sub> )FLSPEHQKAQQRKESKKPPAKLQPR	1.5
3. Ghrelin (1-9)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Glu-His-NH <sub>2</sub>	5.4
4. [Ser <sup>2</sup> (Octanoyl), Ser <sup>3</sup> ]-Ghrelin (1-9)-amide H-Gly-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Ser-Phe-Leu-Ser-Pro-Glu-His-NH <sub>2</sub>	1,100
5. [Ser <sup>2</sup> (Octanoyl)]-Ghrelin (1-9)-amide H-Gly-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Glu-His-NH <sub>2</sub>	1,400
6. [N-Octanoyl, Ser <sup>3</sup> ]-Ghrelin (1-9)-amide C <sub>7</sub> H <sub>15</sub> CO-Gly-Leu-Ser-Phe-Leu-Ser-Pro-Glu-His-NH <sub>2</sub>	>10,000

[0326] The activity was reduced to about 1/200 by transferring an octanoyl group from 3rd serine to 2nd serine in human ghrelin (EC<sub>50</sub> = 1,100 nM).

[0327] The derivative having octanoyl groups at both the 2nd and 3rd positions also showed a reduced activity (EC<sub>50</sub> = 1,400 nM).

[0328] Further, the activity was relatively weakened by N-octanoylation at only the amino-terminal amino group (EC<sub>50</sub> > 10,000 nM).

[0329] From these results, it was revealed that the position of the amino acid modified with an octanoyl group is particularly preferably the 3rd position in the ghrelin molecule.

#### B. Chain length of a fatty acid

[0330] The intracellular Ca-releasing activity of the des-octanoyl derivative derived from rat ghrelin by eliminating the side-chain octanoyl group of 3rd serine was 3,500 nM as compared with the activity (2.6 nM) of the octanoylated ghrelin, and thus it is evident that the side-chain octanoyl group of 3rd serine plays a very important role in expressing the activity.

[0331] Accordingly, the relationship between the activity and the number of carbon atoms in the side-chain acyl group of serine in rat ghrelin was examined using various saturated fatty acids. That is, the intracellular Ca-releasing activities of the ghrelin derivatives wherein the hydroxyl group of 3rd serine was acylated with an acetyl group (CH<sub>3</sub>CO-), propionyl group (CH<sub>3</sub>CH<sub>2</sub>CO-), butyryl group (CH<sub>3</sub>(CH<sub>2</sub>)<sub>2</sub>CO-), hexanoyl group (CH<sub>3</sub>(CH<sub>2</sub>)<sub>4</sub>CO-), decanoyl group (CH<sub>3</sub>(CH<sub>2</sub>)<sub>8</sub>CO-), lauroyl group (CH<sub>3</sub>(CH<sub>2</sub>)<sub>10</sub>CO-), and palmitoyl group (CH<sub>3</sub>(CH<sub>2</sub>)<sub>14</sub>CO-) were determined.

[0332] The results are summarized in Table 5.

Table 5

## Ghrelin derivative activity 2

Compound Structure	Ca-releasing activity EC <sub>50</sub> (nM)
7. [Ser <sup>3</sup> ]-rat Ghrelin GSSFLSPEHQKAQQRKESKKPPAKLQPR	3,500
8. [Ser <sup>3</sup> (Acetyl)]-rGhrelin GSS(CO-CH <sub>3</sub> )FLSPEHQKAQQRKESKKPPAKLQPR	780
9. [Ser <sup>3</sup> (Propionyl)]-rGhrelin GSS(CO-C <sub>2</sub> H <sub>5</sub> )FLSPEHQKAQQRKESKKPPAKLQPR	n.t.
10. [Ser <sup>3</sup> (Butyryl)]-rGhrelin GSS(CO-C <sub>3</sub> H <sub>7</sub> )FLSPEHQKAQQRKESKKPPAKLQPR	280
11. [Ser <sup>3</sup> (Hexanoyl)]-rGhrelin GSS(CO-C <sub>5</sub> H <sub>11</sub> )FLSPEHQKAQQRKESKKPPAKLQPR	16
12. [Ser <sup>3</sup> (Decanoyl)]-rGhrelin GSS(CO-C <sub>9</sub> H <sub>19</sub> )FLSPEHQKAQQRKESKKPPAKLQPR	1.7
13. [Ser <sup>3</sup> (Lauroyl)]-rGhrelin GSS(CO-C <sub>11</sub> H <sub>23</sub> )FLSPEHQKAQQRKESKKPPAKLQPR	2.4
14. [Ser <sup>3</sup> (Palmitoyl)]-rGhrelin GSS(CO-C <sub>15</sub> H <sub>31</sub> )FLSPEHQKAQQRKESKKPPAKLQPR	6.5

[0333] In the table, "n.t." indicates that the sample was not tested.

[0334] The influence of the chain length of fatty acid on the activity was made increasingly significant with an EC<sub>50</sub> value of 780 nM for the ghrelin derivatives having acetyl group (C2) and an EC<sub>50</sub> value of 280 nM for the ghrelin derivatives having butanoyl group (C4), and the ghrelin derivatives having the hexanoyl group (C7) brought about a further increase in the Ca-releasing activity (EC<sub>50</sub> value, 16 nM), and the ghrelin the octanoyl group permitted the Ca-releasing activity to reach a peak (EC<sub>50</sub> value, 1.5 nM). Even the ghrelin derivatives having the decanoyl group (C10) maintained a similar Ca-releasing activity (EC<sub>50</sub> value, 1.7 nM) to that of ghrelin, and further the EC<sub>50</sub> value was 2.4 nM for the ghrelin derivatives having lauroyl group (C12) and 6.5 nM for the ghrelin derivatives having palmitoyl group (C16), thus indicating that the Ca-releasing activity was maintained even if the chain length of fatty acid was increased.

## C. Substitution of various acyl groups

[0335] Human ghrelin derivatives were prepared by binding 3-phenyl propionic acid (HO-CO-CH<sub>2</sub>CH<sub>2</sub>Ph) as a typical example of aromatic fatty acid, 3-octenoic acid (CH<sub>3</sub>(CH<sub>2</sub>)<sub>3</sub>CH=CH-CH<sub>2</sub>COH) as a typical example of unsaturated fatty acid or 4-methyl pentanoic acid ((CH<sub>3</sub>)<sub>2</sub>CH-CH<sub>2</sub>CH<sub>2</sub>CO<sub>2</sub>H) as a typical example of branched fatty acid, in place of saturated fatty acid, via an ester linkage to the hydroxyl group of 3rd serine, and their activity was examined.

## D. Conversion into alkyl groups

[0336] By converting the chemically instable ester linkage into a chemically stable ether or thioether linkage or the like, chemically stable ghrelin derivatives can be formed. However, it goes without saying that maintenance of the activity is a preposition for this conversion.

[0337] Hence, an ether derivative of human ghrelin wherein 3rd serine was octylated (C<sub>8</sub>H<sub>17</sub>) and a thioether derivative of rat ghrelin wherein 3rd serine was replaced by cysteine and octylated were examined for their activity.

[0338] Further, a derivative of human ghrelin wherein 3rd serine was benzylated (-CH<sub>2</sub>Ph) and a derivative of human ghrelin wherein 3rd serine was replaced by cysteine and tritylated (-C(Ph)<sub>3</sub>) were prepared.

[0339] The results are summarized in Table 6. The Ca-releasing activities of the derivative of human ghrelin wherein 3rd serine was benzylated (-CH<sub>2</sub>Ph) and the derivative of human ghrelin wherein 3rd serine was replaced by cysteine and tritylated (-C(Ph)<sub>3</sub>) are shown as those of Compounds 67 and 68 respectively in Table 13. The Ca-releasing activity of the derivative of human ghrelin wherein 4-methyl pentanoic acid ((CH<sub>3</sub>)<sub>2</sub>CH-CH<sub>2</sub>CH<sub>2</sub>CO<sub>2</sub>H) was bound via an ester

linkage to the hydroxyl group of 3rd serine is also shown as that of Compound 69 in Table 13.

Table 6

## Ghrelin derivative activity 3

Compound Structure	Ca-releasing activity EC <sub>50</sub> (nM)
15. [Ser <sup>3</sup> (3-Phenylpropionyl)]-hGhrelin GSS(CO-CH <sub>2</sub> CH <sub>2</sub> Ph) FLSPEHQRVQQRKESKKPPAKLQPR	1.4
16. [Ser <sup>3</sup> (3-Octenoyl)]-hGhrelin GSS(CO-CH <sub>2</sub> CH=CH(CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub> ) FLSPEHQRVQQRKESKKPPAKLQPR	1.7
17. [Ser <sup>3</sup> (Octyl)]-hGhrelin GSS(C <sub>8</sub> H <sub>17</sub> ) FLSPEHQRVQQRKESKKPPAKLQPR	1.2
18. [Cys <sup>3</sup> (Octyl)]-rGhrelin GSC(C <sub>8</sub> H <sub>17</sub> ) FLSPEHQKAQQRKESKKPPAKLQPR	5.4

[0340] Introduction of 3-octenoyl group as an example of unsaturated fatty acid into the side-chain of 3rd serine brought about a similar Ca-releasing activity (EC<sub>50</sub> = 1.7 nM) to the activity of the ghrelin derivatives having an octanoyl group.

[0341] Interestingly, even if a phenyl propionyl group was introduced, the Ca-releasing activity was maintained to be high (EC<sub>50</sub> = 1.4 nM), and even if a 4-methylpentanoyl group (C6) as an example of branched fatty acid was introduced, the EC<sub>50</sub> value was 4.4 nM, indicating that the Ca-releasing activity was maintained (Compound 69 in Table 13), thus revealing that it is not always necessary that the side-chain acyl group in 3rd serine is a linear-chain alkanoyl group.

[0342] Further, the EC<sub>50</sub> values of the ether and thioether derivatives expectable to be chemically stable, wherein 3rd serine or 3rd cysteine were octylated, were maintained to be 1.2 nM and 5.4 nM respectively, thus revealing that it is not always necessary that the side chain of amino acid residue at 3rd position is an acyl group.

[0343] Further, the EC<sub>50</sub> values of ghrelins wherein amino acid residue 3rd position was replaced by Ser (Bzl) [that is, the derivative of human ghrelin wherein 3rd serine was benzylated (-CH<sub>2</sub>Ph)] or by Cys (Trt) [that is, the derivative of human ghrelin wherein 3rd serine was replaced by cysteine and tritylated (-C(Ph)<sub>3</sub>)] were 7.6 nM and 20 nM, respectively, thus indicating that the Ca-releasing activity was maintained (Compounds 67 and 68 in Table 13).

## (2) Determination of the active region

[0344] The intracellular Ca-releasing activity of ghrelin (16-28) containing the original carboxyl-terminal region was relatively low (EC<sub>50</sub> > 10,000 nM), while the EC<sub>50</sub> values of human ghrelin (1-15) and rat ghrelin (1-15) both containing the original amino-terminal region were 7.0 nM and 8.6 nM respectively, thus indicating that the intracellular Ca-releasing activity was maintained, and it was thereby revealed that the active site of ghrelin is present in the amino-terminal region (Table 7).

Table 7

## Ghrelin derivative activity 4

Compound Structure	Ca- releasing activity EC <sub>50</sub> (nM)
19. Ghrelin (16-28) H-Lys-Glu-Ser-Lys-Lys-Pro-pro-Ala-lysLeu-Gln-Pro-Arg-OH	>10,000
20. hGhrelin (1-15) H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-OH	7.0
21. rGhrelin (1-15) H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Glu-His-Gln-Lys-Ala-Gln-Gln-Arg-OH	8.6
22. [des Gln <sup>14</sup> ]-rGhrerin GSS(CO-C <sub>7</sub> H <sub>15</sub> )FLSPEHQKAQ_RKESKKPPAKLQPR	1.5

[0345] Further, because the activities of human and rat ghrelins (1-15) were almost the same, the amino acid residues 11th and 12th position (arginyl-valyl- in human, and -lysyl-aranyl- in rat) are not limited to these amino acids.

[0346] The results of the correlation between structure and activity, obtained using human or rat ghrelin, can be applied to rat and human ghrelins respectively.

[0347] Further, [des-glutamine<sup>14</sup>]-rat ghrelin prepared by removing 14th glutamine from the ghrelin exhibited a Ca-releasing activity (EC<sub>50</sub> = 1.5 nM) similar to that of rat ghrelin, indicating that the amino acid in the middle of the ghrelin molecule may be deleted.

(3) Peptide chain length and introduction of basic group into the carboxyl-terminal

[0348] On the basis of ghrelin (1-15) found to have a relatively strong activity, a derivative was prepared by suitably deleting carboxyl-terminal amino acid residues from the ghrelin(1-15), and their activity was evaluated.

[0349] The activities of the short-chain derivatives having carboxylic acid at the carboxyl-terminal and the short-chain derivatives amidated at the carboxyl-terminal are shown in Table 8.

Table 8

## Ghrelin derivative activity 5

Compound Structure	Ca- releasing activity EC <sub>50</sub> (nM)
23. hGhrelin (1-11) H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-OH	15
24. rGhrelin (1-11) H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Glu-His-Gln-Lys-OH	15
25. Ghrelin (1-10) H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Glu-His-Gln-OH	19
26. Ghrelin (1-9) H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Glu-His-OH	38
27. Ghrelin (1-8) H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Glu-OH	100
28. Ghrelin (1-8)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Glu-NH <sub>2</sub>	13
29. Ghrelin (1-7)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-NH <sub>2</sub>	2.6
30. Ghrelin (1-6)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-NH <sub>2</sub>	4.8
31. Ghrelin (1-5) H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-OH	68
32. Ghrelin (1-5)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-NH <sub>2</sub>	6.2
33-1. Ghrelin (1-4) H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-OH	480
33-2. Ghrelin (1-4)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-NH <sub>2</sub>	160
34. Ghrelin (1-3)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-NH <sub>2</sub>	>10,000

[0350] The Ca-releasing activity of ghrelin (1-3) amide was relatively low (EC<sub>50</sub> > 10,000 nM). The EC<sub>50</sub> of ghrelin (1-4) added phenylalanine to the ghrelin(1-3) was 480 nM and the EC<sub>50</sub> of the carboxyl-terminal amide derivative thereof was 160 nM, thus revealing that they have a significant Ca-releasing activity.

[0351] Further, the activity of ghrelin (1-5) amide added leucine amide to ghrelin(1-4) was about 26 times (EC<sub>50</sub> = 6.2 nM) as high as that of ghrelin(1-4) amide, thus exhibiting a Ca-releasing activity at the same level as that of natural ghrelin.

[0352] The highest Ca-releasing activity was found in ghrelin (1-7) amide, and its EC<sub>50</sub> value was almost equivalent to that of natural ghrelin.

[0353] From the above result, the structural factor essential for expressing the ghrelin activity could be attributed to the sequence of 4th amino-terminal residues, but because its affinity for ghrelin receptor or signal transduction is drastically improved by adding a residue such as leucine at the 5th position, a residue such as leucine is preferably



added at the 5th position.

[0354] As is evident from the above result, the Ca-releasing activity tended to be increased by amidation of carboxyl-terminal carboxylic acid.

[0355] For example, the Ca-releasing activity ( $EC_{50} = 5.4$  nM) of ghrelin (1-9) after amidation was about 7 times as high as the activity ( $EC_{50} = 38$  nM) before amidation, and the Ca-releasing activity ( $EC_{50} = 160$  nM) of ghrelin (1-4) after amidation was about 3 times as high as the activity ( $EC_{50} = 480$  nM) before amidation. Further, the Ca-releasing activity ( $EC_{50} = 13$  nM) of ghrelin (1-8)amide produced from ghrelin (1-9)amide by removing basic histidine residue 9 was lower than the activity ( $EC_{50} = 5.4$  nM) of ghrelin (1-9)amide, while the Ca-releasing activity ( $EC_{50} = 2.6$  nM) of ghrelin (1-7) amide produced by removing glutamic acid 8 as acidic amino acid was higher than the activity ( $EC_{50} = 13$  nM) before removal.

[0356] One effect of amidation is to neutralize the negative charge of carboxylic acid, and the above result indicates that the basicity of carboxyl-terminal amino acid in the short-chain derivative contributes significantly to the increase in activity.

[0357] On the basis of this result, derivatives endowed with basicity at the carboxyl-terminal, which are similar to ghrelin (1-7) amide showing high activity, were prepared and their activity was examined.

[0358] The results are shown in Table 9.

Table 9

Ghrelin derivative activity 6

Compound Structure	Ca-releasing activity $EC_{50}$ (nM)
35. [Lys <sup>6</sup> ]-Ghrelin (1-8)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Lys-NH <sub>2</sub>	1.1
36. [Arg <sup>6</sup> ]-Ghrelin (1-8)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Arg-NH <sub>2</sub>	1.1
37. [Lys <sup>6</sup> ]-Ghrelin (1-6)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Lys-NH <sub>2</sub>	12
38. [Lys <sup>5</sup> ]-Ghrelin (1-5)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Lys-NH <sub>2</sub>	10
39. [ <sup>D</sup> Phe <sup>4</sup> , Lys <sup>5</sup> ]-Ghrelin (1-5)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )- <sup>D</sup> Phe-Lys-NH <sub>2</sub>	1,700

[0359] The Ca-releasing activity ( $EC_{50} = 12$  nM) of [lysine<sup>6</sup>]-ghrelin (1-6) amide having lysine added at the carboxyl-terminal of ghrelin (1-5) was slightly lower than the activity ( $EC_{50} = 4.8$  nM) of ghrelin (1-5), while the Ca-releasing activity ( $EC_{50} = 10$  nM) of ghrelin (1-4) having lysine added at the carboxyl-terminal was about 50 times as high as the activity ( $EC_{50} = 480$  nM) before addition. Further, the Ca-releasing activity ( $EC_{50} = 1.1$  nM), respectively, of the amide derivative having arginine or lysine added at the carboxyl-terminal of ghrelin (1-7) was very stronger than the activity ( $EC_{50} = 2.6$  nM) of ghrelin (1-7) amide.

[0360] It was revealed that in almost all the cases, the activity is increased by masking of acidity at the carboxyl-terminal and introduction of a basic group.

(4) Amino-terminal glycine and 2nd serine residue

[0361] On the basis of ghrelin (1-7) amide ( $EC_{50} = 2.6$  nM) [Compound 29 in Table 8] or ghrelin (1-9) amide ( $EC_{50} = 5.4$  nM) [Compound 3 in Table 4] found to have activity, the influence on the activity of amino-terminal glycine and 2nd serine was examined.

[0362] The results are summarized in Table 10.

Table 10

## Ghrelin derivative activity 7

Compound Structure	Ca-releasing activity EC <sub>50</sub> (nM)
40. [ <i>N</i> -Aminopentanoyl]-Ghrelin (3-7)-amide NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>4</sub> -CO-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-NH <sub>2</sub>	3.4
41. [ <i>N</i> -Acetyl]-Ghrelin (1-10) CH <sub>3</sub> CO-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-Glu-His-Gln-OH	>10,000
42. [ <i>N</i> -Tyr]-rGhrelin YGSS(CO-C <sub>7</sub> H <sub>15</sub> )FLSPEHQKAQQRKESKKPPAKLQPR	120
43. [ <i>N</i> -Glycyl]-Ghrelin (3-7)-amide H-Gly-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-NH <sub>2</sub>	380
44. [Leu <sup>2</sup> ]-Ghrelin (1-7)-amide H-Gly-Leu-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-NH <sub>2</sub>	42
45. [His <sup>2</sup> ]-Ghrelin (1-7)-amide H-Gly-His-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-NH <sub>2</sub>	35
46. [Lys <sup>2</sup> ]-Ghrelin (1-7)-amide H-Gly-Lys-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-NH <sub>2</sub>	24
47. [Gly <sup>2</sup> ]-Ghrelin (1-7)-amide H-Gly-Gly-Ser(CO-C <sub>7</sub> H <sub>15</sub> )-Phe-Leu-Ser-Pro-NH <sub>2</sub>	78

[0363] The activity of *N*<sup>α</sup>-acetyl-ghrelin (1-10) wherein the amino-terminal amino group in the ghrelin(1-10) was blocked was relatively low (EC<sub>50</sub> > 10,000 nM). As described above, the activity of [*N*<sup>α</sup>-octanoyl, serine<sup>3</sup>]-ghrelin (1-9) amide (Compound 6 in Table 1) was also relatively low (EC<sub>50</sub> > 10,000 nM), and thus the amino-terminal amino group is preferably not blocked in order to express the Ca-releasing activity.

[0364] On the other hand, the Ca-releasing activity of *N*<sup>α</sup>-aminopentanoyl-ghrelin (3-7) amide wherein amino-terminal glycine and 2nd serine were replaced by 5-amino-n-pentanoic acid (NH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-CO-) having a length of 2nd residues was almost maintained (EC<sub>50</sub> = 3.4 nM), the Ca-releasing activity of [*N*<sup>α</sup>-glycyl]-ghrelin (3-7) amide from which 2nd serine had been deleted was lower (EC<sub>50</sub> = 380 nM), and the Ca-releasing activity of [*N*-tyrosyl]-rat ghrelin having a tyrosine residue added at the amino-terminal in the rat ghrelin was lower (EC<sub>50</sub> = 120 nM), so that it is preferable, for obtaining stronger activity, that the amino-terminal amino group is present at a position of a length of 2 residues from octanoyl 3rd serine residue to the amino-terminal.

[0365] Further, the EC<sub>50</sub> values of the derivatives of ghrelin (1-7) amide wherein 2nd serine had been replaced by leucine, glycine, histidine and lysine were 42 nM, 78 nM, 24 nM and 35 nM respectively, indicating a slightly lower Ca-releasing activity than that of ghrelin (1-7) amide.

[0366] Since this result indicates that the 2nd serine residue (-NH-CH(CH<sub>2</sub>OH)-CO-) can be replaced by the partial structure -CH<sub>2</sub>-CH<sub>2</sub>-CO- in aminopentanoic acid, the 2nd serine residue acts at least as a spacer for separating amino-terminal amino group of ghrelin by a predetermined distance from the 3rd octanoyl group. The reason that the activity was maintained by replacement of the 2nd serine residue by 5-aminopentanoic acid is that the basicity of the amino-terminal was increased by introducing its alkylamine structure.

[0367] In summary, the amino group of amino-terminal glycine residue is considered to confer basicity on the amino-terminal of ghrelin molecule, thus expressing the activity of ghrelin, and therefore the amino group at the amino-terminal is preferably not blocked.

[0368] Further, the 2nd serine residue is considered to act as a spacer for separating the amino group at the amino-terminal by a predetermined distance from the 3rd octanoyl group, and therefore the 2nd serine residue may be replaced by an amino acid or non-amino acid compound having a relatively less bulky side chain. That is, the position of the

octanoyl group in the ghrelin molecule is defined relative to the amino-terminal amino group, and this positional relationship constitutes a part of the active structure of ghrelin.

[0369] That is, the side chain of 2nd amino acid is preferably relatively less bulky such as in serine, alanine and norvaline rather than an amino acid having a bulky structure, and an amino acid residue not restricting the flexibility of neighboring residues is preferable as the 2nd amino acid. Further, because the Ca-releasing activity of N<sup>α</sup>-aminopentanoyl-ghrelin (3-7) amide is almost maintained (EC<sub>50</sub> = 3.4 nM), 2nd serine can be replaced by a non-amino acid compound.

(5) Optical activity of 3rd and 4th amino acid residues

[0370] On the basis of the structure of ghrelin (1-7) amide, its derivatives wherein 3rd L-serine and 4th L-phenylalanine had been replaced by the corresponding L-amino acids were prepared, and the influence which the configuration of 3rd and 4th amino acid have on the Ca-releasing activity was examined. Specifically, on the basis of [serine<sup>3</sup> (octyl)]-ghrelin (1-7) amide (EC<sub>50</sub> = 5.8 nM) [Compound 50 in Table 11] and [cysteine<sup>3</sup> (octyl)]-ghrelin (1-7) amide (EC<sub>50</sub> = 7.4 nM) [Compound 48 in Table 11] which maintained a good activity, their derivatives wherein 3rd serine and 4th phenylalanine had been replaced by the corresponding L- or D-amino acids were prepared.

[0371] The results are summarized in Table 11. From these results, both 3rd and 4th amino acids are preferably L-amino acids.

Table 11

Ghrelin derivative activity 8

Compound structure	Ca-releasing activity EC <sub>50</sub> (nM)
48. [Cys <sup>3</sup> (Octyl)]-Ghrelin (1-7)-amide H-Gly-Ser-Cys(C <sub>8</sub> H <sub>17</sub> )-Phe-Leu-Ser-Pro-NH <sub>2</sub>	7.4
49. [Cys <sup>3</sup> (Octyl), <sup>D</sup> Phe <sup>4</sup> ]-Ghrelin (1-7)-amide H-Gly-Ser-Cys(C <sub>8</sub> H <sub>17</sub> )- <sup>D</sup> Phe-Leu-Ser-Pro-NH <sub>2</sub>	3,000
50. [Ser <sup>3</sup> (Octyl)]-Ghrelin (1-7)-amide H-Gly-Ser-Ser(C <sub>8</sub> H <sub>17</sub> )-Phe-Leu-Ser-Pro-NH <sub>2</sub>	5.8
51. [Ser <sup>3</sup> (Octyl), <sup>D</sup> Phe <sup>4</sup> ]-Ghrelin (1-7)-amide H-Gly-Ser-Ser(C <sub>8</sub> H <sub>17</sub> )- <sup>D</sup> Phe-Leu-Ser-Pro-NH <sub>2</sub>	2,200
52. [ <sup>D</sup> Ser <sup>3</sup> (Octyl)]-Ghrelin (1-7)-amide H-Gly-Ser- <sup>D</sup> Ser(C <sub>8</sub> H <sub>17</sub> )-Phe-Leu-Ser-Pro-NH <sub>2</sub>	>10,000
53. [ <sup>D</sup> Ser <sup>3</sup> (Octyl), <sup>D</sup> Phe <sup>4</sup> ]-Ghrelin (1-7)-amide H-Gly-Ser- <sup>D</sup> Ser(C <sub>8</sub> H <sub>17</sub> )- <sup>D</sup> Phe-Leu-Ser-Pro-NH <sub>2</sub>	>10,000

(6) Mode of linkage of a side chain at the 3rd position

[0372] Derivatives of ghrelin wherein the original ester linkage was replaced by an ester in the reverse direction (Compound No. 54), an amide (Compound Nos. 55 and 56), a disulfide (Compound No. 57) and methylene (Compound No. 58) were prepared such that the side chain at the 3rd position became the same length as that of the ghrelin chain (-CH<sub>2</sub>-O-CO-C<sub>7</sub>H<sub>15</sub>). In addition, ester derivatives having steric hindrance on the β-carbon atom of amino acid at the 3rd position (Compound Nos. 59 and 60) and an amide derivative wherein 3 methylene units had been extended (Compound No. 61) were prepared. The results are summarized in Table 12.

Table 12

## Ghrelin derivative activity 9

Compound structure	Activity EC <sub>50</sub> (nM)
54. [Asp <sup>3</sup> (O-Heptyl)]-hGhrelin GSD(O-C <sub>7</sub> H <sub>15</sub> )FLSPEHQRVQQRKESKKPPAKLQPR	5.1
55. [Asp <sup>3</sup> (NH-Heptyl)]-hGhrelin GSD(NH-C <sub>7</sub> H <sub>15</sub> )FLSPEHQRVQQRKESKKPPAKLQPR	11
56. [Dap <sup>3</sup> (Octanoyl)]-hGhrelin GS-NH- <sup>L</sup> CH(CH <sub>2</sub> NHCO-C <sub>7</sub> H <sub>15</sub> )-CO-FLSPEHQRVQQRKESKKPPAKLQPR	2.6
57. [Cys <sup>3</sup> (S-Heptyl)]-hGhrelin GSC(S-C <sub>7</sub> H <sub>15</sub> )FLSPEHQRVQQRKESKKPPAKLQPR	1.4
58. [Adod <sup>3</sup> ]-hGhrelin GS-NH-CH(n-C <sub>10</sub> H <sub>21</sub> )-CO-FLSPEHQRVQQRKESKKPPAKLQPR	0.91
59. [Thr <sup>3</sup> (Octanoyl)]-hGhrelin GST(CO-C <sub>7</sub> H <sub>15</sub> )FLSPEHQRVQQRKESKKPPAKLQPR	10
60. [Leu <sup>3</sup> , Thr <sup>3</sup> (Octanoyl)]-hGhrelin GLT(CO-C <sub>7</sub> H <sub>15</sub> )FLSPEHQRVQQRKESKKPPAKLQPR	46
61. [Lys <sup>3</sup> (Octanoyl)]-hGhrelin GSK(CO-C <sub>7</sub> H <sub>15</sub> )FLSPEHQRVQQRKESKKPPAKLQPR	32

[0373] The activity of Compound 58, wherein the side chain at the 3rd position had been replaced by methylene units exclusively, showed the strongest activity (EC<sub>50</sub> value = 1 nM or less). The activity of other derivatives was varied depending on the type of linkage, but it was confirmed that the mode of linkage of a side chain of amino acid 3 does not exert a significant influence on the activity.

(7) Hydrophobicity of a side chain at the 3rd position

[0374] Derivatives wherein Ser (octanoyl) group 3 had been replaced by a hydrophobic amino acid most of which are natural amino acids was prepared, and their activity was examined. The results are summarized in Table 13.

Table 13

## 10. Activity of Ghrelin derivatives

Compound Structure	Activity EC <sub>50</sub> (nM)
62. [Trp <sup>3</sup> ]-hGhrelin GSWFLSPEHQRVQQRKESKKPPAKLQPR	31
63. [Phe <sup>3</sup> ]-hGhrelin GSFFLSPEHQRVQQRKESKKPPAKLQPR	2,000
64. [Cha <sup>3</sup> ]-hGhrelin GS-Cha-FLSPEHQRVQQRKESKKPPAKLQPR	19
65. [2-Nal <sup>3</sup> ]-hGhrelin GS-Nal-FLSPEHQRVQQRKESKKPPAKLQPR	8.2
66. [2-DNal <sup>3</sup> ]-hGhrelin GS-DNal-FLSPEHQRVQQRKESKKPPAKLQPR	>10,000
67. [Ser <sup>3</sup> (Bzl)]-hGhrelin GSS(CH <sub>2</sub> -C <sub>6</sub> H <sub>5</sub> )FLSPEHQRVQQRKESKKPPAKLQPR	7.6
68. [Cys <sup>3</sup> (Trityl)]-hGhrelin GSC(C-Ph <sub>3</sub> )FLSPEHQRVQQRKESKKPPAKLQPR	20
69. [Ser <sup>3</sup> (4-Methylpentanoyl)]-hGhrelin GSS(CO-CH <sub>2</sub> CH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub> )FLSPEHQRVQQRKESKKPPAKLQPR	4.4
70. [Leu <sup>3</sup> ]-hGhrelin GSLFLSPEHQRVQQRKESKKPPAKLQPR	4,400
71. [Ile <sup>3</sup> ]-hGhrelin GSIFLSPEHQRVQQRKESKKPPAKLQPR	>10,000
72. [Lys <sup>3</sup> ]-hGhrelin GSKFLSPEHQRVQQRKESKKPPAKLQPR	120
73. [Nle <sup>3</sup> ]-hGhrelin GS-Nle-FLSPEHQRVQQRKESKKPPAKLQPR	2,800
74. [Val <sup>3</sup> ]-hGhrelin GSVFLSPEHQRVQQRKESKKPPAKLQPR	1,600

[0375] The EC<sub>50</sub> values of the derivatives having an aromatic hydrophobic amino acid such as tryptophan, cyclohexyl alanine or naphthyl alanine at the 3rd position were 31 nM, 19 nM and 8.2 nM respectively, indicating that the Ca-releasing activity was maintained. Unexpectedly, when phenyl alanine was introduced at the 3rd position, the Ca-releasing activity was slightly low, but even if more hydrophobic Ser (Bzl) or Cys (Trityl) was introduced at the 3rd position, the Ca-releasing activity was similarly maintained, and thus it was confirmed that the hydrophobicity of the side chain at the 3rd position is more preferable for expressing the activity.

[0376] On one hand, when an aliphatic hydrophobic amino acid such as leucine, isoleucine, norleucine or valine was introduced at the 3rd position, the Ca-releasing activity of the derivatives was generally maintained but slightly lower than the derivatives introducing the aromatic amino acid. The activity of Compound 73 having norleucine at the 3rd position was EC<sub>50</sub> = 2,800 nM, whereas the activity of 6-amino-norleucine (lysine; Compound 72) having an amino group added to a side chain of norleucine was increased to 120 nM in terms of EC<sub>50</sub> values, so it was confirmed that similar to the basicity of carboxyl-terminal described above, the basicity of a side chain at the 3rd position is also preferable.

## (8) Short-chain ghrelin derivatives

[0377] As described above, it was found that a ghrelin fragment of amino-terminal amino acids 1 to 4 shows significant activity and this activity is further increased by adding leucine at 5th position to said fragment; 3rd amino acid residue is preferably the one having a hydrophobic side chain; the activity is increased by introducing a basic residue; and amino acid residues 1 and 2 may be replaced by a non-amino acid compound having a 2-residue length, such as  $\delta$ -amino acid. On the basis of these results, various short-chain ghrelin derivatives based on the amino-terminal region (1-5) were prepared as shown in Compound Nos. 76 to 87 in Tables 14 and 15, and their activities were examined. The results are summarized in Tables 14 and 15.

[0378] Compound 80 is known (Ipamorelin; K. Raum et al., Eur. J. of Endocrinol., 139: 552-561, 1998).

Table 14

## Ghrelin derivative activity 11

Compound structure	Activity EC <sub>50</sub> (nM)
75. [Lys <sup>1</sup> ]-Ghrelin (1-7)-amide H-Gly-Ser-Ser(CO-C <sub>7</sub> H <sub>13</sub> )-Phe-Leu-Ser-Lys-NH <sub>2</sub>	11
76. [N-Aminopentanoyl, Ser <sup>3</sup> (Octyl), Lys <sup>5</sup> ]-Ghrelin (3-5)-amide NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>4</sub> -CO-Ser(C <sub>8</sub> H <sub>17</sub> )-Phe-Lys-NH <sub>2</sub>	12
77. [N-Aminopentanoyl, <sup>D</sup> Ser <sup>3</sup> (Octyl), <sup>D</sup> Phe <sup>4</sup> , Lys <sup>5</sup> ]-Ghrelin (3-5)-amide NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>4</sub> -CO- <sup>D</sup> Ser(C <sub>8</sub> H <sub>17</sub> )- <sup>D</sup> Phe-Lys-NH <sub>2</sub>	1,600
78. [Aib <sup>1</sup> , His <sup>2</sup> , Ser <sup>3</sup> (Octyl), Lys <sup>5</sup> ]-Ghrelin (1-5)-amide H-Aib-His-Ser(C <sub>8</sub> H <sub>17</sub> )-Phe-Lys-NH <sub>2</sub>	34
79. [Aib <sup>1</sup> , His <sup>2</sup> , <sup>D</sup> Ser <sup>3</sup> (Octyl), <sup>D</sup> Phe <sup>4</sup> , Lys <sup>5</sup> ]-Ghrelin (1-5)-amide H-Aib-His- <sup>D</sup> Ser(C <sub>8</sub> H <sub>17</sub> )- <sup>D</sup> Phe-Lys-NH <sub>2</sub>	38
80. [Aib <sup>1</sup> , His <sup>2</sup> , <sup>D</sup> Nal <sup>3</sup> , <sup>D</sup> Phe <sup>4</sup> , Lys <sup>5</sup> ]-Ghrelin (1-5)-amide H-Aib-His- <sup>D</sup> Nal- <sup>D</sup> Phe-Lys-NH <sub>2</sub>	2.5

[0379] Since the Ca-releasing activity of known Compound 80 was high (2.5 nM), the activity of Compound 79 derived from Compound 80 by replacing 2-D-naphthyl alanine at the 3rd position by D-octyl serine was also examined, and as a result, its EC<sub>50</sub> value was 38 nM, indicating that the activity was maintained. Compound 77 having D-octyl serine and D-phenyl alanine at the 3rd and 4th positions, which has the same amino acid structure as that of Compound 79 except for amino acids 1 and 2, showed a lower activity (1,600 nM), and these results indicate that the sequence or structure of amino acids 1 and 2 also affects the steric configuration of side chains of 3rd and 4th amino acids important for exhibiting the activity.

[0380] That is, in the case where amino acids 1 and 2 were replaced by aminopentanoic acid, the activity was kept at 34 nM even if 2-D-naphthyl alanine at the 3rd position and D-phenyl alanine at the 4th position were replaced by their corresponding L-amino acids (Compound 78), and thus the amino acid sequence (Gly-Ser) at the 1st to 2nd positions in ghrelin requires L configuration for amino acids 3 and 4, but even if amino acids 3 and 4 have D configuration, the activity is made significant by introducing another amino acid sequence such as Aib-His. It was also confirmed that regardless of L- or D-configuration at the 3rd and 4th positions, the activity is expressed by introduction of aminopentanoic acid at the 1st and 2nd positions.

Table 15

## Ghrelin derivative activity 12

Compound Structure	Activity EC <sub>50</sub> (nM)
81. [ <i>N</i> -Aminopentanoyl, Ser <sup>3</sup> (Octyl)]-Ghrelin (3-5)-amide NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>4</sub> -CO-Ser(C <sub>8</sub> H <sub>17</sub> )-Phe-Leu-NH <sub>2</sub>	11
82. [ <i>N</i> -Aminopentanoyl, Ser <sup>3</sup> (Octyl)]-Ghrelin (3-5)-methanamide NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>4</sub> -CO-Ser(C <sub>8</sub> H <sub>17</sub> )-Phe-Leu-NH-CH <sub>3</sub>	12
83. [ <i>N</i> -Aminopentanoyl, Ser <sup>3</sup> (Octyl)]-Ghrelin (3-5)-ethanamide NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>4</sub> -CO-Ser(C <sub>8</sub> H <sub>17</sub> )-Phe-Leu-NH-C <sub>2</sub> H <sub>5</sub>	22
84. [ <i>N</i> -Aminopentanoyl, Ser <sup>3</sup> (Octyl)]-Ghrelin (3-5)-benzamide NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>4</sub> -CO-Ser(C <sub>8</sub> H <sub>17</sub> )-Phe-Leu-NH-CH <sub>2</sub> -C <sub>6</sub> H <sub>5</sub>	98
85. [ <i>N</i> -Aminopentanoyl, Ser <sup>3</sup> (Octyl)]-Ghrelin (3-5), aminoethanamide NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>4</sub> -CO-Ser(C <sub>8</sub> H <sub>17</sub> )-Phe-Leu-NH-(CH <sub>2</sub> ) <sub>2</sub> -NH <sub>2</sub>	3.5
86. [ <i>N</i> -Aminopentanoyl, Ser <sup>3</sup> (Octyl), MePhe <sup>4</sup> , MeLeu <sup>5</sup> ]-Ghrelin (3-5)-amide NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>4</sub> -CO-Ser(C <sub>8</sub> H <sub>17</sub> )-MePhe-MeLeu-NH <sub>2</sub>	82
87. [ <sup>125</sup> I]-hGhrelin GSS(CO-C <sub>7</sub> H <sub>13</sub> )F- <sup>125</sup> I-SPEHQRVQQRKESKKPPAKLQPR	220

[0381] Using [*N*-Aminopentanoyl, Ser<sup>3</sup>(Octyl)]-Ghrelin (3-5) based on the amino-terminal region (1-5) of ghrelin, the correlation between activity and structure in the carboxyl-terminal region was examined. The activity of its derivatives wherein carboxyl-terminal leucine at the 5th position had been modified with amide, methyl amide, ethyl amide or benzyl amide was maintained but tended to be decreased as shown by EC<sub>50</sub> values of 11 nM, 12 nM, 22 nM and 98 nM, respectively. On the other hand, by replacing ethyl amide by aminoethyl amide, the activity was increased as shown by an EC<sub>50</sub> value of 3.5 nM, thus revealing that impartment of basicity to the carboxyl-terminal of ghrelin molecule is preferable.

[0382] These various carboxyl-terminal amide derivatives are useful compounds because of their resistance *in vivo* to decomposition with carboxy peptidases. Compound 86 (EC<sub>50</sub> = 86 nM) containing N-methylamino acid is also an useful compound because of its resistance to the enzymes.

Example 12. GH releasing activity of ghrelin derivatives in rat

(1) GH-releasing activity of various long-chain ghrelin derivatives in rat

[0383] 18 nmol/kg Compound 17 ([Ser<sup>3</sup>(Octyl)]-hGhrelin), 30 nmol/kg Compound 18 ([Cys<sup>3</sup>(Octyl)]-rGhrelin), 100 nmol/kg Compound 65 ([2-<sup>125</sup>I]-hGhrelin), or 18 nmol/kg Compound 15 ([Ser<sup>3</sup>(3-Phenylpropyl)]-hGhrelin) was administered rapidly and intravenously into IGS-SD strain rats (about 7-week-old) under anesthesia with Nembutal for each sample (n = 3). Fifteen minutes after administration, plasma was collected, and the concentration of GH in plasma was measured by radioimmunoassay (Biotrak/Amersham). Separately, 0.2 % bovine serum albumin (BSA)-physiolog-

ical saline, 6 nmol/kg rGhrelin and hGhrelin, or 80 nmol/kg Ipamorelin (Compound 80) was administered into other rats as the control, respectively, and the concentrations of GH in plasma in 15 minutes after administration were compared (for each sample,  $n = 3$ ).

**[0384]** The results are shown in Table 13. Compound 17 ([Ser<sup>3</sup>(Octyl)]-hGhrelin), Compound 18 ([Cys<sup>3</sup>(Octyl)]-rGhrelin) and Compound 15 ([Ser<sup>3</sup>(3-PhPr)]-hGhrelin) exhibited a strong GH-releasing activity, and the GH-releasing activity of [2-<sup>14</sup>NaI<sup>3</sup>]-hGhrelin showed a good correlation with intracellular Ca-releasing activity.

Table 16

GH-releasing activity of various long-chain ghrelin derivatives						
Compound administered	EC <sub>50</sub> value (nM)	dose (nmol/kg)	GH level in plasma in 15 min. after administration (ng/mL)			
			Rat 1	Rat 2	Rat 3	M±S.D.
Physiological saline	-	-	32	52	59	49±12
hGhrelin	1.3	6	1802	1613	2203	1873±301
rGhrelin	1.5	6	2056	1082	1205	1448±530
Ipamorelin (Compound 80)	2.5	80	377	260	1184	607±503
[Ser <sup>3</sup> (Octyl)]-hGhrelin	1.2	18	1626	1602	1743	1657±75
[Cys <sup>3</sup> (Octyl)]-hGhrelin	5.4	30	2786	2342	2354	2494±253
[Ser <sup>3</sup> (phenylpropionyl)]-hGhrelin	1.4	18	2119	2078	1581	1926±299
[2- <sup>14</sup> NaI <sup>3</sup> ]-hGhrelin	8.2	100	1637	1576	1357	1524±147

(2) Change of GH in plasma by administration of (Cys<sup>3</sup>(Octyl)]-rat ghrelin

**[0385]** After Compound 18 ([Cys<sup>3</sup>(Octyl)]-rat Ghrelin) was intravenously administered in a dose of 5 µg/head into Wistar strain male rats (about 260 to 280 g) under anesthesia with Nembutal, GH released to blood was measured. Physiological saline as the control and rat Ghrelin (5 µg/head) were also administered and compared with Compound 18.

**[0386]** As shown in Tables 17 to 19, the GH secretion-promoting activity of [Cys<sup>3</sup>(Octyl)]-rat Ghrelin was equivalent to natural rat ghrelin (that is, C<sub>max</sub> of secreted GH was about 1,100 ng/ml for both ghrelins), and further the secretion time tended to prolong. The intracellular Ca-releasing activity of Compound 18 was 5.4 nM in terms of EC<sub>50</sub>.



Table 17

Change of GH level in plasma by administration of  
[Cys<sup>3</sup>(Octyl)]-rat ghrelin

[Cys(C18) <sup>3</sup> ]-rat ghrelin 5 µg/head		Time (min)						
		0	5	10	15	20	30	60
GH level in plasma (ng/mL)	Rat 1	377	338	687	927	900	469	98
	Rat 2	101	294	258	300	358	245	86
	Rat 3	59	476	949	1229	1417	704	133
	Rat 4	33	530	959	1451	1299	800	220
	Rat 5	32	613	1060	1561	1359	726	122
Mean		120	450	783	1093	1067	589	132
±S.D.		±146	±133	±324	±506	±445	±229	±53

Table 18

Change of GH level in plasma by administration of physiological  
saline

Physiologic al saline		Time (min)						
		0	5	10	15	20	30	60
GH level in plasma (ng/mL)	Rat 1	0	88	129	133	116	107	430
	Rat 2	204	122	118	134	128	69	36
	Rat 3	77	0	0	0	0	0	11
	Rat 4	0	0	0	0	48	27	110
	Rat 5	0	0	0	0	0	0	210
Mean		56	42	49	53	58	41	159
±S.D.		±89	±58	±67	±73	±61	±47	±170

Table 19

Change of GH level in plasma by administration of rat ghrelin

Rat ghrelin 5 µg/head		Time (min)						
		0	5	10	15	20	30	60
GH level in plasma (ng/mL)	Rat 1	143	186	425	405	215	56	3
	Rat 2	10	1396	2028	1566	876	242	27
	Rat 3	838	163	443	681	419	120	36
	Rat 4	348	556	1387	1469	1293	663	100
	Rat 5	0	875	1380	1009	1414	452	20
Mean		268	635	1133	1026	843	306	37
±S.D.		±348	±517	±690	±498	±525	±250	±37

## Example 13. Increasing action of ghrelin on appetite

## (1) Appetite-increasing action by administration into ventricle

[0387] Physiological saline containing rat ghrelin at various concentrations was administered at 8 : 45 a.m. into cerebral ventricles of male Wistar strain rats (16 to 20 animals per group) each weighing 300 to 325 g. As the control, ghrelin-free physiological saline was administered into ventricles. After administration, the rats were allowed feed *ad libitum*, and the amount of feed taken for 2 hours after administration was measured. As shown in Fig. 6, an increase in the amount of feed taken was observed in the rats administered 50pmol ghrelin intracerebroventricularly, and a dose-dependent increase in the amount of feed taken was observed in the rat administered 200 pmol and 500 pmol ghrelin, but the amount of feed taken was decreased in the rats administered 2nmol ghrelin. Usually, the rat takes feed at night, so that in the morning, the rat is on a full stomach and rarely takes feed (see the rat administered physiological saline as the control in Fig. 6), and thus the increase in the amount of taken feed by administering ghrelin into cerebral ventricle indicates that ghrelin has an appetite-increasing action.

## (2) Appetite-increasing action by intravenous administration

[0388] 50 µg/kg rat ghrelin was intravenously administered into tail veins in 9-month-old male SD (Sprague-Dawley) rats (5 animals) and Wister rats (4 animals), and the amount of feed taken for 2 hours after administration was measured (evaluated during 16 : 00 to 19 : 00 p.m.). As shown in Table 20, the amount of feed taken was evidently increased by intravenous administration of ghrelin, as compared with the amount of feed taken without administration of rat ghrelin, which was determined using the same animal at the same hour on another day. That is, it was demonstrated that ghrelin has an appetite-increasing action even by intravenous administration.

Table 20

strain	Rat No.	Amount of feed taken (g)	
		Administration of ghrelin	No administration of ghrelin
S-D	1	3.2	2.2
	2	3.7	1.0
	3	3.2	0.1
	4	2.7	1.3
	5	2.6	0.8
	Mean	3.1	1.1
	S.D.	0.4	0.8
Wister	6	2.3	0.2
	7	1.9	1.4
	8	1.6	0.1
	9	2.1	0.3
	Mean	2.0	0.5
	S.D.	0.3	0.6

## Example 14. Enhancement of gastric functions by ghrelin

[0389] To examine the effect of ghrelin on gastric functions, the following experiment was carried out. Male SD strain rats (7- to 8-week-old, weighing 200 to 280 g) were fasted for 20 hours or more and then used in the experiment. The rats were anesthetized by intraperitoneal administration of urethane (1.25 g/kg) and kept warm using a warming pad and a warming light. A tracheal canula was inserted, and the esophagus was ligated by silk thread, and each rat was subjected to the following operation in order to measure gastric acid secretion or gastric motility. In the experiment using conscious animal, the rat was subjected to the operation for measurement of gastric acid secretion or gastric

mobility under slight anesthesia by inhalation of ether.

[0390] In the experiment for gastric acid secretion under anesthesia with urethane, the operation was conducted according to the method proposed by Ohno et al. [Ohno, T., et al., *Jpn. J. Pharmacol.* **43**, 429-439 (1987)]. Briefly, in the supine position the abdomen was incised and the stomach and duodenum were exposed. A polyethylene tube was inserted into a front part of the stomach to prepare acute stomach fistula. Another polyethylene tube was inserted into the stomach after cleaving the duodenum, and the surrounding part of the pylorus was ligated and fixed. The inside of the stomach was infused with physiological saline which was adjusted to pH 7.0 in a reservoir and warmed at 37 °C. The flow rate was 1.0 ml/min. The infusion fluid was adjusted to pH 7.0 by titration with 100 mM NaOH using a pH-fixing unit (Hiranuma, Comitite-8). After it was confirmed that a basic amount of gastric acid secretion was stable, the test chemical was intravenously administered, and the rate of secretion of gastric acid was measured at 5-minute intervals. Four rats were used in each group.

[0391] In the experiment during arousal, the rat was subjected to the same operation under slight anesthesia by inhalation of ether, and then a small cut was made in the flank, and an infusion tube was taken out from the body. The exposed stomach and duodenum were put back in the abdomen, and the excised site was sutured, and the animal was fastened while lying on the back in a Ballman-type rat-fixing cage, and after it was confirmed that the rat was recovered from anesthesia, the rat was subjected to the experiment. The esophagus was ligated, but a trachea canula was not inserted.

[0392] The experiment for measurement of stomach motility under urethane anesthesia, a miniaturized balloon method was used according to the method proposed by Takeuchi & Nobuhara [Takeuchi, K. and Nobuhara, Y., *Digestive Diseases and Sciences* **30**, 1181-1188 (1985)]. That is, a balloon filled with water and a supporting catheter were inserted into the stomach after cleavage of a front part of the stomach. It was fixed to lie on a gland of the stomach line, and one end of the catheter was connected to a pressure transducer (LPU-0.1-350-0-II, from Nihon Kohoden Corporation). After it was confirmed that the gastric motility was stable, the test chemical was intravenously administered accumulatively at 60-minute intervals. For the gastric motility, the amplitude of internal pressure in the stomach and the number of shrinking reactions in shrinkage motility having an amplitude of 20 cm H<sub>2</sub>O or more were measured at 10-minute intervals. Four animals were used in each group. In the experiment using conscious animals, the rat was subjected to the same operation under light anesthesia by inhalation of ether, and after the excised site was sutured, the animal was fastened in the prone position in a Ballman-type rat-fixing cage. After it was confirmed that the rat was recovered from anesthesia, the animal was subjected to the experiment.

[0393] Rat ghrelin and histamine dihydrochloride were dissolved in physiological saline and administered in a dose of 1 ml/kg into tail vein. To examine whether the vagus nerve action is involved in the action of ghrelin, atropine sulfate was subcutaneously administered in 30 minutes before administration of ghrelin, or the cervical vagus nerve bundles were bilaterally cut off. To examine the involvement of histamine H<sub>2</sub> receptor in the action of ghrelin, famotidine (Gaster®, produced by Yamanouchi Pharmaceutical Co., Ltd.) was subcutaneously administered 30 minutes before administration of ghrelin. The results are shown in mean ± standard error. Statistical analysis was performed using Dunnett's multiple comparison tests. P value < 0.05 was judged to be statistically significant.

[0394] As shown in Fig. 7A and in Table 21, secretion of gastric acid was promoted in a dose-dependent manner upon intravenous administration of rat ghrelin in a dose of 0.8 to 20 µg/kg into the rat under urethane anesthesia.

[0395] In the rat under anesthesia, the spontaneous motility of stomach was hardly observed before administration of ghrelin. When rat ghrelin was intravenously administered in a dose of 0.8 to 20 µg/kg into the rat in this condition, both the amplitude and frequency of gastric motility were promoted as shown in Fig. 8A & B and in Table 21. These reactions were observed immediately after administration of rat ghrelin. By administration of 20 µg/kg, secretion of gastric acid was increased and reached to the maximum level within 20 minutes and gradually decreased for 90 minutes after administration. As shown in Fig. 7A & B, the maximum reaction in the gastric acid secretion-promoting action by administration of 20 µg/kg rat ghrelin was almost comparable to the reaction induced by intravenous administration of 3 mg/kg histamine. The action of promoting the amplitude of gastric motility reached the maximum reaction within 10 minutes in any dose, and by administration of 20 µg/kg ghrelin, the action was gradually decreased until 50 minutes after administration.

[0396] Further, as shown in Table 21, the action of promoting gastric secretion induced by administration of 20 µg/kg rat ghrelin was inhibited almost completely by pretreatment with atropine or bilateral cervical vasotomy, but this action was not affected by pretreatment of subcutaneous administration of 1 mg/kg famotidine i.e. a histamine H<sub>2</sub> receptor antagonist. Further, the action of promoting gastric motility induced by administration of rat ghrelin was completely inhibited by pretreatment with atropine or bilateral cervical vagotomy. From these results, it was confirmed that the promoting action of ghrelin on gastric functions is not via histaminergic mechanism but via activation of vagus nerve system.

[0397] By intravenous administration of rat ghrelin (4 and 20 µg/kg), secretion of gastric acid was promoted in the conscious rat in the same way as in the rat under urethane anesthesia. As compared with the rat under anesthesia, the conscious rat had spontaneous gastric motility before administration of the test chemical, and even in this condition

by administering 0.8 to 20 µg/kg rat ghrelin into the rat, the gastric motility was promoted together with its amplitude and frequency. From the above result, it was confirmed that by intravenous administration of ghrelin, promotion of gastric acid secretion and promotion of gastric motility occur not only in the anesthetized rat but also in the conscious rat.

Table 21

Treatment		Gastric acid secretion (µ equivalent /60 min)	Gastric motility	
			Frequency (times /60min.)	Amplitude (cm H <sub>2</sub> O /60 min.)
Physiological saline		17.6±1.2	1.3±1.0	1.7±1.0
Rat ghrelin	0.8 µg/kg i.v. injection	24.5±2.2	35.5±18.1	6.7±4.4
	4 µg/kg i.v. injection	23.5±2.6	60.8±25.6	11.1±5.3
	20 µg/kg i.v. injection	43.3±4.6 (*1)	100.5±20.4 (*1)	21.8±2.5 (*1)
Intravenous injection of 20 µg/kg rat ghrelin	+ atropine 1 mg/kg subcutaneous administration	26.1±3.9 (*2)	0 (*3)	0 (*3)
	+removal of vagus nerve	18.4±3.7 (*3)	0 (*3)	0 (*3)
	+ famotidine 1 mg/kg subcutaneous administration	43.0±4.2	NT	NT
Symbols in the table indicate: *1, p<0.01; *2, p<0.05; and *3, p<0.01 NT: Not tested.				

Example 15. Promoting action of ghrelin and ghrelin derivatives on cell growth

[0398] To examine the action of administered ghrelin on promotion of cell growth, the following experiment was conducted. Twenty µg/kg of rat ghrelin or thioether-type rat ghrelin (Compound 18 [Cys<sup>3</sup>(octyl)]-hGhrelin) was administered into tail veins of Wister male rats (7.5-week-old) respectively. Seventeen hours after administration, <sup>3</sup>H-thymidine was administered into tail veins, and 1 hour thereafter, duodenum, jejunum and bone marrow were excised. The incorporation of <sup>3</sup>H-thymidine to DNA fractions of these tissues was measured in order to examine the cell growth-promoting action of ghrelin and ghrelin derivatives. The tissues were cut thin and homogenized using a Polytron homogenizer, and after centrifugation, the supernatant was precipitated with trichloroacetic acid to give a DNA fraction. The radioactivity of the DNA fraction was measured by a liquid scintillation counter.

[0399] As shown in Table 22, the incorporation of <sup>3</sup>H-thymidine into these tissues or organs was increased by intravenous administration of rat ghrelin or thioether-type rat ghrelin, and it was thus confirmed that ghrelin exhibits a cell growth-promoting action in duodenum, jejunum and bone marrow.

[0400] The time course of the cell growth-promoting action after intravenous administration of ghrelin was similar to that after administration of GHRH (growth hormone releasing hormone), so it was considered that the cell growth-promoting action of ghrelin occurs via GH (growth hormone) secreted mainly from pituitary. It was considered that the regulation of GH secretion by ghrelin as a physiological factor is reasonable for organism regulation, and there are less adverse effects which could occur by GH administration.

Table 22

	Comparative Example	Rat ghrelin	Thioether-type ghrelin
Bone marrow (in tissues)	100.0 ±17.8%	141.7 ±30.1%	144.5 ±16.5%

Table 22 (continued)

	Comparative Example	Rat ghrelin	Thioether-type ghrelin
Duodenum (in DNA fraction)	100.0 ±14.2%	136.0 ±17.8%	114.0 ±11.7%
Jejunum (in DNA fraction)	100.0 ±6.8 %	159.0 ±7.5%	151.0 ±23.6%

Numerical values show the ratio (%) of incorporation of radioisotope relative to the mean (in triplicate) of the comparative example (i.e. the group given physiological saline).

#### Example 16. Quantification of ghrelin by anti-ghrelin antibody

[0401] Using antibodies raised against amino- and carboxyl-terminal rat ghrelin peptides as antigens, ghrelin in various living tissues was quantified by radioimmunoassay (RIA).

[0402] Rabbits were immunized with [C-Cys]-rat ghrelin [1-11] (rat ghrelin peptide of amino acids 1 to 11 from amino-terminal having cysteine bound to the carboxyl-terminal thereof) and [C-Cys]-rat ghrelin [13-28] (rat ghrelin peptide of amino acids 13 to 28 from amino-terminal having cysteine bound to the carboxyl-terminal thereof) as antigens, to form amino-terminal antibody (anti-[C-Cys]-rat ghrelin [1-11] antigen) and carboxyl-terminal antibody (anti-[C-Cys]-rat ghrelin [13-28] antigen) respectively.

[0403] As shown in Fig. 9a, the  $IC_{50}$  (50% inhibitory concentration) of rat ghrelin was 3.1 fmol in binding between radioisotope-labeled rat ghrelin and the amino-terminal antibody. This amino-terminal antibody, while showing 100 % cross-reactivity with chemically synthesized human ghrelin and rat ghrelin, showed only 0.3 % cross-reactivity with n-hexanoyl rat ghrelin wherein 3rd serine had been modified with n-hexanoyl group and 20 % cross-reactivity with n-decanoyl rat ghrelin wherein 3rd serine had been modified with n-decanoyl group. Further, the amino-terminal antibody did not react with ghrelin from which fatty acid had been released.

[0404] The amino-terminal antibody showed similar affinity for rat ghrelin (28 amino acids), human ghrelin (28 amino acids), and ghrelin-27 (ghrelin consisting of 27 amino acids) found in human and rat. Accordingly, it was confirmed that the amino-terminal antibody specifically recognizes natural ghrelin wherein 3rd serine was modified with n-octanoyl group.

[0405] As shown in Fig. 9b, natural rat ghrelin modified with n-octanoyl group and rat ghrelin modified by removing n-octanoyl from the natural rat ghrelin showed a similar  $IC_{50}$  value of 44 fmol in binding between radioisotope-labeled rat ghrelin and the carboxyl-terminal antibody. That is, it was confirmed that the carboxyl-terminal antibody has the same affinity for ghrelin modified with fatty acid and for ghrelin from which fatty acid was released.

[0406] These results revealed that in regard to ghrelins occurring in various tissues in a living body, ghrelin wherein 3rd serine was modified with n-octanoyl group can be quantified by the amino-terminal antibody, while both ghrelin modified with fatty acid and ghrelin from which fatty acid was released can be quantified by the carboxyl-terminal antibody.

[0407] Table 23 shows the result of examination of the contents of fatty acid-modified ghrelin and the contents of both fatty acid-modified ghrelin and fatty acid-released ghrelin in various tissues in a living body.

Table 23

Tissues	Amount of rat ghrelin reacting with antibody (fmol/mg tissues)	
	C-RIA	N-RIA
Hypothalamus	1.8±0.3	<0.05
Pituitary	8.5±3.1	<0.05
Thyroid	3.5±2.0	<0.05
Mandible gland	8.8±1.3	<0.05
Thymus	3.5±0.4	<0.05
Adrenal gland	3.1±0.4	<0.05
Atrium	2.3±0.2	0.07±0.01
Ventricle	2.1±0.1	<0.05

Table 23 (continued)

Tissues	Amount of rat ghrelin reacting with antibody (fmol/mg tissues)	
Aorta	2.4±0.7	0.14±0.03
Lung	3.1±0.4	<0.05
Liver	2.8±0.5	<0.05
Pancreas	2.6±0.6	0.15±0.05
Stomach	1779.8±533.9	377.31±55.83
Duodenum	106.7±7.3	20.57±0.69
Jejunum	60.2±17.2	10.73±5.44
Ileum	20.5±5.1	0.16±0.08
Cecum	15.1±2.5	1.70±5.44
Colon	10.4±0.7	<0.05
Kidney	5.4±0.3	<0.05
Spermary	2.8±0.2	<0.05
Plasma (1 mL)	219.6±71.8	4.02±1.91

[0408] In the table, C-RIA indicates the result of quantification by radioimmunoassay using the carboxyl-terminal antibody, while N-RIA indicates the result of quantification by the amino-terminal antibody.

[0409] The numerical values in the table indicate "mean±standard deviation".

Example 17. Production of rat ghrelin (1-28) by a semi-synthesis method

#### Synthesis scheme

[0410] In this example, rGhrelin was produced from rGhrelin (6-28) and Ghrelin (1-7) fragments previously prepared by genetic engineering method and chemical synthesis respectively, as follows.

[0411] Specifically,  $\beta$ -galactosidase 97S-(QFE-SRHRR)-rGhrelin (6-28), that is, a fusion protein of  $\beta$ -galactosidase 97S and rGhrelin (6-28) between which an amino acid sequence (-QFE-SRHRR-) having a site cleaved by V8 protease and KexII protease occurred was expressed in *E. coli*. This fusion protein was treated with V8 protease, to cut off SRHRR rGhrelin (6-28). Then, all amino groups of SRHRR rGhrelin (6-28) were protected with Boc groups, and the resulting peptide was treated with KexII protease, to give [Lys(Boc)<sup>11, 16, 19, 20, 24</sup>]-rGhrelin (6-28) from which the amino-terminal amino group of Ser 6 had been isolated. This protected fragment was condensed with [N<sup>α</sup>-Boc]-rGhrelin (1-5)-Osu obtained by chemical synthesis, and the resulting [Lys(Boc)<sup>11, 16, 19, 20, 24</sup>]-rGhrelin was treated with an acid, whereby rGhrelin was produced.

[0412] In this example, semi-synthesis of rGhrelin was described, but hGhrelin can also be synthesized by this method.

[0413] Further, in this example, fragment (1-5) was condensed with fragment (6-28), but chemically synthesized amino-terminal fragments (1-2), (1-3) and (1-7) can be condensed respectively with carboxyl-terminal fragments (3-28), (4-28) and (8-28) of an arbitrary length consisting of amino acids at the 28th position up to the 3rd position constructed by genetic engineering means, in order to produce ghrelin as a fusion protein. To reduce the number of steps in chemical synthesis, the condensation between (1-2) and (3-28) or between (1-3) and (4-28) is advantageous. From the viewpoint of complete prevention of the racemization caused by condensation, the condensation between (1-7) and (8-28) by using Pro 7 is particularly preferable.

Construction of expression vector pG97s rGR and expression of ghrelin (6-28) as a fusion protein

[0414] On the basis of the nucleotide sequence of rat ghrelin cDNA, a DNA fragment for rGhrelin (6-28) having an amino acid sequence QFE-SRHRR in the prepro region was obtained by annealing using a total synthetic oligomer.

[0415] To insert this DNA fragment into pG97SnPPH34 (JP-A 9-296000), pG97SnPPH34 was treated with Sall and SmaI thereby deleting its human parathyroid hormone precursor gene. The product was treated with alkali phosphatase and ligated by T4 ligase to the rGhrelin derivative gene fragment previously treated with Sall and kinase. The ligated

plasmid was transformed into *E. coli* DH5 $\alpha$  strain, to give plasmid pG97s rGR.

[0416] The resulting plasmid pG97s rGR was transformed into *E. coli* M25 (*ompT*), and the resulting transformant was cultured onto 3 dishes each containing 200 ml Terrific broth liquid medium (1.2 % trypton, 2.4 % yeast extract, 0.4 % glucose) and cultured under shaking at 37 °C. When the concentration (OD<sub>660</sub>) of the bacterial cell reached 0.8, isopropyl 1-thio- $\beta$ -D-galactopyranoside (IPTG) was added thereto at a final concentration of 2 mM, to express rGhrelin (6-28) fusion protein. Further, the bacterial cell was cultured for 4 hours and then collected by centrifugation. The structure of rGhrelin (6-28) fusion protein is as follows:

rGhrelin 6-28 fusion protein: ( $\beta$ -Galactosidase-97S)-QFE-SRHRR rGhrelin (6-28)

Processing of rGhrelin 6-28 fusion protein and purification of [SRHRR]-rGhrelin (6-28)

[0417] 20 ml of the resulting bacterial cell was suspended in TE buffer and the bacterial cell was disrupted by a French press. Thereafter, the inclusion body was collected by centrifugation at 3000 rpm for 15 minutes, suspended again in 10 ml TE buffer and deionized water, and centrifuged whereby the inclusion body was washed. The inclusion body was diluted with deionized water such that its OD<sub>660</sub> was reduced to 50.0, and Tris-HCl (pH 8.2) was added thereto at a final concentration of 50 mM, and the inclusion body was dissolved in urea (final concentration: 3.5 M). To this solution kept at 30 °C was added rV8 protease derivative V8D5 (abbreviated hereinafter to V8D5) (JP-A9-47291) at a final concentration of 10  $\mu$ g/ml, and the solution was treated with the enzyme at 30 °C for 20 minutes. The reaction was terminated by adding 3 % acetic acid (AcOH).

[0418] 1.5-fold excess deionized water was added to the V8D5 enzyme reaction-terminated solution containing the [SRHRR]-rGhrelin (6-28), then this solution was adjusted to pH 5.0 with 5N NaOH, to precipitate the  $\beta$ -galactosidase derivative fragment which was then removed by centrifugation at 5000 rpm for 10 minutes.

[0419] The supernatant containing [SRHRR]-rGhrelin (6-28) was applied to TSK-ODS 80Ts column (resin particle diameter of 20  $\mu$ m, 50 mm I.D.  $\times$  100 mm, TOSOH Co., Ltd.) previously equilibrated with 0.1 % TFA. The desired peptide was eluted by a linear gradient of from 100 % buffer A [0.8 ml/min., 1 % acetonitrile, 0.1 % TFA] to 100 % buffer B [50 % acetonitrile, 0.095 % TFA], which was programmed to be finished in a volume of 5 columns. Fractions containing the desired peptide [SRHRR]-rGhrelin (6-28) were collected (about 50 mg).

Purification of [Boc-SRHRR]-[Lys(Boc)<sup>11, 16, 19, 20, 24</sup>]-rGhrelin (6-28)

[0420] 6-equivalent mole (19.2 mg, 6  $\times$  15  $\mu$ mol) of di-*t*-butyl bicarbonate was added to 50 % aqueous acetonitrile solution containing about 50 mg (15  $\mu$ mol) of [SRHRR]-rGhrelin (6-28), then adjusted to pH 9 with triethylamine, and left at room temperature for 15 minutes. Acetic acid was added at a final concentration of 0.5 % to the reaction solution, and after the acetonitrile was evaporated, the solution was added to EMPORE-Octyl (C8) HD 4 mm/1 ml cartridge previously equilibrated with 10 % acetonitrile containing 0.1 % TFA, and after the column was washed with the equilibration solution, [Boc-SRHRR]-[Lys(Boc)<sup>11, 16, 19, 20, 24</sup>]-rGhrelin (6-28) was eluted with 90 % acetonitrile containing 0.095 % TFA. The acetonitrile was evaporated, and 6 ml solution containing about 30 mg of the desired peptide was obtained.

[0421] Mass spectrometry indicated mainly two peptides whose molecular weight after Boc modification was higher by 500 (determined molecular weight, 3895) or by 600 (determined molecular weight, 3995) than the molecular weight (determined molecular weight = 3396, theoretical molecular weight = 3398) before Boc modification.

Cleavage of [Lys(Boc)<sup>11, 16, 19, 20, 24</sup>]-rGhrelin (6-28) by Kex2 protease and purification thereof.

[0422] A calcium chloride solution and Tris-HCl, pH 8.2, were added at final concentrations of 0.3 mM and 20 mM respectively to the resulting aqueous solution of [Boc-SRHRR]-[Lys(Boc)<sup>11, 16, 19, 20, 24</sup>]-rGhrelin (6-28) (30 mg, 6 mL). After a solution of Kex2 protease (JP-A 10-229884) was added thereto at a concentration of 1  $\times$  10<sup>5</sup> units/ml, the sample was treated with the protease at 30 °C for 60 minutes.

[0423] In HPLC, a peak of [Boc-SRHRR]-[Lys(Boc)<sup>11, 16, 19, 20, 24</sup>]-rGhrelin (6-28) disappeared, a peak of [Lys(Boc)<sup>11, 16, 19, 20, 24</sup>]-rGhrelin(6-28) was shifted toward the side of hydrophobicity, and a peak of a hydrophilic fragment corresponding to Boc-SRHRR was observed.

[0424] After the disappearance of the starting material was confirmed, the reaction solution was adjusted to pH 3.5 with aqueous acetic acid and applied to reversed-phase chromatography column ODS-80Ts (column volume of 1.66 cc, resin particle diameter 20  $\mu$ m, TOSOH Co., Ltd.) previously equilibrated with 1.0 % acetonitrile containing 1 % acetic acid. After the column was washed with the equilibration solution in a volume of 5 columns, [Lys(Boc)<sup>11, 16, 19, 20, 24</sup>]-rGhrelin (6-28) was eluted by a linear gradient of from 1.0 % acetonitrile to 90.0 % acetonitrile each containing 1 % acetic acid, which was programmed to be finished in a volume of 5 columns. Main fractions were lyophilized to give 6.2 mg of the desired protected peptide.

## Fragment condensation and de-protection

[0425] Triethylamine (51.0  $\mu$ l, 0.366 mmol) and a solution of di-t-butyl bicarbonate (78.0 mg, 0.0356 mmol) in TFE (4.00 ml) were added respectively to a solution of Ghrelin (1-5) (190 mg, 0.0301 mmol, Compound 31) in trifluoroethanol (TFE) (6.00 ml) and stirred at room temperature for 13 hours. The solvent was evaporated, and ether (20.0 ml) was added to the resulting residues, whereby 180.5 mg [ $N^{\alpha}$ -Boc]-rGhrelin (1-5) was obtained.

[0426] Then, HOSu (5.20 mg, 0.0452 mmol) was added to a solution of [ $N^{\alpha}$ -Boc]-rGhrelin (1-5) (22.0 mg, 0.0301 mmol) in DMF (1.00 ml), and DIPCl (7.30  $\mu$ l, 0.0466 mmol) was added thereto in a bath at -30 °C. After the mixture was stirred in the bath at -30 °C for 1 hour and then at room temperature for 18 hours, the solvent was evaporated, and the resulting residues were converted into powder with ether to give 14.1 mg [ $N^{\alpha}$ -Boc]-rGhrelin (1-5)-OSu as a succinimide ester of [ $N^{\alpha}$ -Boc]-rGhrelin (1-5).

[0427] Then, [ $N^{\alpha}$ -Boc]-rGhrelin (1-5)-OSu (3.3 mg, 3.96  $\mu$ mol) and triethylamine (2.5  $\mu$ l, 17.9  $\mu$ mol) were added to a solution in DMF (0.6 ml) of [Lys(Boc)<sup>11,16,19,20,24</sup>]-rGhrelin (6-28) (6.10 mg, 2.18  $\mu$ mol) prepared by the recombinant method and stirred at room temperature for 24 hours. The solvent was evaporated, and TFA (2.00 ml) was added directly to the resulting residues under cooling on ice and stirred at room temperature for 1.5 hours. The TFA was evaporated, and ether was added to the residues, whereby 6.2 mg crude peptide containing Ghrelin (1-28) was obtained.

[0428] This product was dissolved in 2 ml of 5 % acetic acid (AcOH) and applied to YMC-Pack-ODS-A (5  $\mu$ m, 20 mm $\times$ 250 mm) and eluted by a linear gradient (flow rate: 10 ml/min.) of from 0 to 95 % acetonitrile in 0.1% trifluoroacetic acid for 60 minutes. The desired fractions were collected, lyophilized, applied to YMC-Pack PROTEIN-RP (C4, 10 mm $\times$ 250 mm) and eluted by a linear gradient (flow rate: 4.7 ml/min.) of from 7.5 to 21.3 % acetonitrile in 0.1% trifluoroacetic acid for 30 minutes.

[0429] The desired fractions were collected, lyophilized and applied to YMC-Pack PROTEIN-RP (C4, 10 mm $\times$ 250 mm) and eluted by a linear gradient (flow rate: 4.7 ml/min.) of from 7.5 to 21.3% acetonitrile in 0.1% trifluoroacetic acid for 30 minutes. The desired fractions were collected and lyophilized to give 2.1 mg rGhrelin (1-28). This product showed a retention time agreeing with that of standard rGhrelin (1-28) in analytical HPLC, and had an intracellular Ca-releasing activity of  $EC_{50}=1.5$  nM which was equivalent to natural ghrelin.

ESI-MS 3315.0 (theoretical: 3314.8), amino acid composition: Ser; 3.74 (4), Glx; 5.69 (6), Gly; 1.18 (1), Ala; 2.05 (2), Leu; 2, Phe; 0.98 (1), Lys; 4.98 (5), His; 1.03(1), Arg; 1.96 (2), Pro; 4.01 (4)

Compound 87 [ $^{15}P$ ]-rGhrelin (1-28)

[0430] As a by product in succinimide esterification of [ $N^{\alpha}$ -Boc]-rGhrelin (1-5) or condensation of the fragments, 0.8 mg [ $^{15}P$ ]-rGhrelin (1-28) was obtained. Its intracellular Ca-releasing activity was  $EC_{50} = 220$  nM.

ESI-MS 3315.0 (theoretical: 3314.8), amino acid composition: Ser; 3.80 (4), Glx; 5.92 (6), Gly; 1.23 (1), Ala; 2.07 (2), Leu; 2, Phe; 0.97 (1), Lys; 4.92 (5), His; 1.02 (1), Arg; 1.97 (2), Pro; 4.11 (4)

GC-MS analysis of leucine after hydrolysis in  $D_2O/DCI$ : L-Leu; 1.17 (1), D-Leu; 0.83 (1)

## Industrial Applicability

[0431] By administering the new peptide-type compound of the present invention or a pharmaceutically acceptable salt thereof into humans or animals, it demonstrates an excellent working effect as a pharmaceutical preparation for promoting growth of children and ameliorating the defect of metabolic functions caused by GH deficiency, by inducing GH secretion without causing substantial side effects, and its antibody demonstrates an excellent working effect as an agent for diagnosis of diseases attributable to GH deficiency and as a research tool in the field of science.



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His Gly Gln Pro Leu Gly Lys Phe Leu Gln Asp Ile Leu Trp Glu Glu

95 100 105

30 gtc act gag gcc ccg gcc gac aag tgattgtccc tgagaccagc 379

Val Thr Glu Ala Pro Ala Asp Lys

35 110 115

40 cacctctgtt ctcccagcct cctaagggct cacctggctt ccaggacgct tccactatca 439

caccagctc tgagggatgc tagcctggga ggtgaataaa cattcagact gg 491

&lt;210&gt; 22

&lt;211&gt; 27

45

&lt;212&gt; PRT

&lt;213&gt; Bos taurus

&lt;223&gt; Amino acid sequence for bovine endogenous peptides (27 amino acids) of growth hormone secretagogue

50

&lt;400&gt; 22

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Glu Leu Gln Arg Lys Glu

55 1 5 10 15

Ala Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg

20

25

5

<210> 23

<211> 89

10

<212> PRT

<213> Bos taurus

15

<223> Partial amino acid sequence for a prepro-form of bovine endogenous peptides (27 amino acids) of growth hormone secretagogue

<400> 23

20

Asp Leu Ala Met Ala Gly Ser Ser Phe Leu Ser Pro Glu His Gln Glu  
1 5 10 15

Leu Gln Arg Lys Glu Ala Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg  
20 25 30

25

Thr Leu Glu Gly Gln Phe Asp Phe Glu Val Gly Ser Gln Ala Glu Gly  
35 40 45

Ala Glu Asp Glu Leu Glu Ile Arg Phe Asn Ala Phe Phe Asn Ile Gly  
50 55 60

30

Ile Lys Leu Ala Gly Ala Gln Ser Leu Gln His Gly Gln Thr Leu Gly  
65 70 75 80

Lys Phe Leu Gln Asp Ile Leu Trp Glu

35

85

<210> 24

<211> 267

40

<212> DNA

<220>

<221> CDS

45

<222> (1)... (267)

<213> Bos taurus

50

<223> Base sequence of cDNA coding prepro-form of bovine endogenous peptides (27 amino acids) of growth hormone secretagogue

<400> 24

55

gac ttg gcc atg gcg ggc tcc agc ttt ctg agc ccc gaa cat cag gaa 48  
Asp Leu Ala Met Ala Gly Ser Ser Phe Leu Ser Pro Glu His Gln Glu

1                      5                      10                      15  
 ctg cag aga aag gaa gct aag aag cca tca ggc aga ctg aag ccc cgg 96  
 5 Leu Gln Arg Lys Glu Ala Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg  
                     20                      25                      30  
 acc ctg gaa ggc cag ttt gac ccg gag gtg gga agt cag gcg gaa ggt 144  
 10 Thr Leu Glu Gly Gln Phe Asp Phe Glu Val Gly Ser Gln Ala Glu Gly  
                     35                      40                      45  
 gca gag gac gag ctg gaa atc cgg ttc aac gcc ccc ttt aac att ggg 192  
 15 Ala Glu Asp Glu Leu Glu Ile Arg Phe Asn Ala Phe Phe Asn Ile Gly  
                     50                      55                      60  
 atc aag cta gca ggg gct cag tcc ctc cag cat ggc cag acg ttg ggg 240  
 20 Ile Lys Leu Ala Gly Ala Gln Ser Leu Gln His Gly Gln Thr Leu Gly  
                     65                      70                      75                      80  
 aag ttt ctt cag gac atc ctc tgg gaa 267  
 Lys Phe Leu Gln Asp Ile Leu Trp Glu  
 25                      85

<210> 25

<211> 24

<212> PRT

<213> Gallus domesticus

<223> Amino acid sequence for chicken endogenous peptides of  
 growth hormone secretagogue

<400> 25

Gly Ser Ser Phe Leu Ser Pro Thr Tyr Lys Asn Ile Gln Gln Gln Lys

1                      5                      10                      15

Gly Thr Arg Lys Pro Thr Ala Arg

20

<210> 26

<211> 21

<212> PRT

<213> Anguilla japonica

<220>

<221> AMIDATION

<222> 21



<223> Amino acid sequence for eel endogenous peptides of growth hormone secretagogue

<400> 26

Gly Ser Ser Phe Leu Ser Pro Ser Gln Arg Pro Gln Gly Lys Asp Lys

1 5 10 15

Lys Pro Pro Arg Val

20

<210> 27

<211> 28

<212> PRT

<213> Rana cafesbeiana

<223> Amino acid sequence for frog endogenous peptides of growth hormone secretagogue

<400> 27

Gly Leu Ser Phe Leu Ser Pro Ala Glu Met Gln Lys Ile Ala Glu Arg

1 5 10 15

Gln Ser Gln Asn Lys Leu Arg His Gly Asn Met Arg

20

25

<210> 28

<211> 27

<212> PRT

<213> Xenopus laevis

<223> Amino acid sequence for frog (Xenopus laevis) endogenous peptides of growth hormone secretagogue

<400> 28

Gly Leu Thr Phe Leu Ser Pro Ala Asp Met Gln Lys Ile Ala Glu Arg

1 5 10 15

Gln Ser Gln Asn Lys Leu Arg His Gly Asn Met

20

25

<210> 29

<211> 23

<212> PRT

<213> Oncorhynchus mykiss

<220>

<221>AMIDATION

5 <222> 23

<223> Amino acid sequence for rainbow trout endogenous peptides (23 amino acids) of growth hormone secretagogue

10 <400> 29

Gly Ser Ser Phe Leu Ser Pro Ser Gln Lys Pro Gln Val Arg Gln Gly

1 5 10 15

15 Lys Gly Lys Pro Pro Arg Val

20

<210> 30

20 <211> 20

<212> PRT

<213> *Oncorhynchus mykiss*

25 <220>

<221> AMIDATION

<222> 20

30 <223> Amino acid sequence for rainbow trout endogenous peptides (20 amino acids) of growth hormone secretagogue

<400> 30

Gly Ser Ser Phe Leu Ser Pro Ser Gln Lys Pro Gln Gly Lys Gly Lys

35 1 5 10 15

Pro Pro Arg Val

20

40 <210> 31

<211> 28

45 <212> PRT

<213> *Canis familiaris*

<223> Amino acid sequence for dog endogenous peptides of growth hormone secretagogue

50 <400> 31

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Gln Arg Lys

1 5 10 15

55 Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg

20

25

5

&lt;210&gt; 32

&lt;211&gt; 108

&lt;212&gt; PRT

10

<213> *Anguilla japonica*

<223> Amino acid sequence for prepro-form of eel endogenous  
peptides of growth hormone secretagogue

15

&lt;400&gt; 32

Met Lys Arg Thr Ala Tyr Ile Ile Leu Leu Val Cys Val Leu Ala Leu

1 5 10 15

20

Trp Met Asp Ser Val Gln Ala Gly Ser Ser Phe Leu Ser Pro Ser Gln

20 25 30

Arg Pro Gln Gly Lys Asp Lys Lys Pro Pro Arg Val Gly Arg Arg Asp

35 40 45

25

Ser Asp Gly Ile Leu Asp Leu Phe Met Arg Pro Pro Leu Gln Asp Glu

50 55 60

Asp Ile Arg His Ile Thr Phe Asn Thr Pro Phe Glu Ile Gly Ile Thr

30

65 70 75 80

Met Thr Glu Glu Leu Phe Gln Gln Tyr Gly Glu Val Met Gln Lys Ile

85 90 95

35

Met Gln Asp Leu Leu Met Asp Thr Pro Ala Lys Glu

100 105

&lt;210&gt; 33

40

&lt;211&gt; 114

&lt;212&gt; PRT

<213> *Xenopus laevis*

45

<223> Amino acid sequence frog (*Xenopus laevis*) endogenous  
peptides of growth hormone secretagogue

&lt;400&gt; 33

50

Met Asn Phe Gly Lys Ala Ala Ile Phe Gly Val Val Leu Phe Cys Leu

1 5 10 15

Leu Trp Thr Glu Gly Ala Gln Ala Gly Leu Thr Phe Leu Ser Pro Ala

20 25 30

55

Asp Met Gln Lys Ile Ala Glu Arg Gln Ser Gln Asn Lys Leu Arg His

35                      40                      45  
 Gly Asn Met Asn Arg Arg Gly Val Glu Asp Asp Leu Ala Gly Glu Glu  
 5                      50                      55                      60  
 Ile Gly Val Thr Phe Pro Leu Asp Met Lys Met Thr Gln Glu Gln Phe  
 65                      70                      75                      80  
 10 Gln Lys Gln Arg Ala Ala Val Gln Asp Phe Leu Tyr Ser Ser Leu Leu  
 85                      90                      95  
 Ser Leu Gly Ser Val Gln Asp Thr Glu Asp Lys Asn Glu Asn Pro Gln  
 15                      100                      105                      110  
 Ser Gln

20 <210> 34  
 <211> 82  
 <212> PRT  
 <213> Oncorhynchus mykiss  
 25 <223> Amino acid sequence for prepro-form of rainbow trout  
 endogenous peptides (23 amino acids) of growth hormone  
 secretagogue

30 <400>34  
 Met Ile Leu Met Leu Cys Thr Leu Ala Leu Trp Ala Lys Ser Val Ser  
 1                      5                      10                      15  
 Ala Gly Ser Ser Phe Leu Ser Pro Ser Gln Lys Pro Gln Val Arg Gln  
 35                      20                      25                      30  
 Gly Lys Gly Lys Pro Pro Arg Val Gly Arg Arg Asp Ile Glu Ser Phe  
 35                      40                      45  
 40 Ala Glu Leu Phe Glu Gly Pro Leu His Gln Glu Asp Lys His Asn Thr  
 50                      55                      60  
 Ile Lys Ala Pro Phe Glu Met Gly Ile Thr Met Ser Glu Glu Glu Phe  
 45                      65                      70                      75                      80  
 Gln Glu

50 <210> 35  
 <211> 99  
 <212> PRT  
 <213> Oncorhynchus mykiss  
 55 <223> Amino acid sequence for prepro-form of rainbow trout

endogenous peptides (20 amino acids) of growth hormone  
secretagogue

5

<400>35

Met Ile Leu Met Leu Cys Thr Leu Ala Leu Trp Ala Lys Ser Val Ser

1

5

10

15

10

Ala Gly Ser Ser Phe Leu Ser Pro Ser Gln Lys Pro Gln Gly Lys Gly

20

25

30

Lys Pro Pro Arg Val Gly Arg Arg Asp Ile Glu Ser Phe Ala Glu Leu

35

40

45

15

Phe Glu Gly Pro Leu His Gln Glu Asp Lys His Asn Thr Ile Lys Ala

50

55

60

Pro Phe Glu Met Gly Ile Thr Met Ser Glu Glu Glu Phe Gln Glu Tyr

20

65

70

75

80

Gly Ala Val Leu Gln Lys Ile Leu Gln Asp Val Leu Gly Asp Thr Ala

85

90

95

25

Thr Ala Glu

<210> 36

30

<211> 503

<212> DNA

<220>

<221> CDS

35

<222> (66)... (389)

<213> Anguilla japonica

40

<223> Base sequence of cDNA coding prepro-form of eel  
endogenous peptides of growth hormone secretagogue

<400> 36

tccaagagggc actgggtttc ctcttaaagt gcaaaactcc actgtgagct tcagacatga 60

45

ggcag atg aaa cgc acc gca tac atc atc ctg ctg gtc tgc gtc ctg 107

Met Lys Arg Thr Ala Tyr Ile Ile Leu Leu Val Cys Val Leu

1

5

10

50

gcg ctg tgg atg gac tct gtc cag gct ggc tcc agc ttc ctc agc ccc 155

Ala Leu Trp Met Asp Ser Val Gln Ala Gly Ser Ser Phe Leu Ser Pro

15

20

25

30

55

tca cag aga ccg cag ggg aag gat aag aag cct ccc agg gtt ggc aga 203

Ser Gln Arg Pro Gln Gly Lys Asp Lys Lys Pro Pro Arg Val Gly Arg  
 35 40 45  
 5 cga gac tca gat ggg atc ctg gac ctg ttt atg agg ccc cca ttg cag 251  
 Arg Asp Ser Asp Gly Ile Leu Asp Leu Phe Met Arg Pro Pro Leu Gln  
 50 55 60  
 10 gat gaa gac atc aga cac att acg ttt aac act cct ttt gag atc ggg 299  
 Asp Glu Asp Ile Arg His Ile Thr Phe Asn Thr Pro Phe Glu Ile Gly  
 65 70 75  
 15 atc acc atg act gag gag ctg ttc cag caa tat gga gaa gtg atg cag 347  
 Ile Thr Met Thr Glu Glu Leu Phe Gln Gln Tyr Gly Glu Val Met Gln  
 80 85 90  
 aag atc atg cag gat ttg ctg atg gac aca cct gcc aaa gag 389  
 20 Lys Ile Met Gln Asp Leu Leu Met Asp Thr Pro Ala Lys Glu  
 95 100 105  
 tgacaagagt ggatgatc tggacttcac aaaaccctgc gtcccatata ttcctgcatt 449  
 25 attgcatgca taattcaacc aattgttaa catttaataa aattttgcaa acgc 503  
  
 <210> 37  
 <211> 484  
 30 <212> DNA  
 <220>  
 <221> CDS  
 35 <222> (47)... (388)  
 <213> *Xenopus laevis*  
 <223> Base sequence of cDNA coding prepro-form of frog  
 (Xenopus laevis) endogenous peptides of growth hormone  
 40 secretagogue  
 <400> 37  
 tttcactttt atctcgagg cggcaccggt gaccaggacc ttcagg 46  
 45  
 atg aat ttt ggt aaa gcc gcc atc ttt ggg gtt gtc ttg ttc tgc ctg 94  
 Met Asn Phe Gly Lys Ala Ala Ile Phe Gly Val Val Leu Phe Cys Leu  
 1 5 10 15  
 50 ctg tgg acg gag ggg gcc cag gct ggc ttg acc ttc ctg agt cca gcc 142  
 Leu Trp Thr Glu Gly Ala Gln Ala Gly Leu Thr Phe Leu Ser Pro Ala  
 20 25 30  
 55

gac atg cag aag att gcg gag agg caa tca cag aat aag ctg aga cac 190  
 Asp Met Gln Lys Ile Ala Glu Arg Gln Ser Gln Asn Lys Leu Arg His  
 5 35 40 45  
 ggc aat atg aat cgc agg ggt gtg gag gat gac ctg gcc ggg gag gag 238  
 Gly Asn Met Asn Arg Arg Gly Val Glu Asp Asp Leu Ala Gly Glu Glu  
 10 50 55 60  
 atc ggg gtg acc ttc cct ctg gat atg aag atg acg cag gag cag ttc 286  
 Ile Gly Val Thr Phe Pro Leu Asp Met Lys Met Thr Gln Glu Gln Phe  
 65 70 75 80  
 15 cag aag cag agg gct gcg gtg cag gac ttc ctg tac tcc tcc ctc ctc 334  
 Gln Lys Gln Arg Ala Ala Val Gln Asp Phe Leu Tyr Ser Ser Leu Leu  
 85 90 95  
 20 tct ctc ggg tca gtg cag gat aca gaa gac aag aat gaa aat cct cag 382  
 Ser Leu Gly Ser Val Gln Asp Thr Glu Asp Lys Asn Glu Asn Pro Gln  
 100 105 110  
 25 agc caa tgagaatgat gaaaatccgc tctgtctctga tgccctccc cgatctgtgt 438  
 Ser Gln  
 30 gtctttatta tctctgtgta acccagaaat aaatcttatt tatggc 484  
 <210> 38  
 <211> 462  
 35 <212> DNA  
 <220>  
 <221> CDS  
 40 <222> (12)... (257)  
 <213> Oncorhynchus mykiss  
 <223> Base sequence of cDNA coding prepro-form of rainbow trout  
 endogenous peptides (23 amino acids) of growth hormone  
 45 secretagogue  
 <400> 38  
 50 tcacaggtct c atg ata ctg atg ctg tgt act ctg gct ctg tgg gcc 47  
 Met Ile Leu Met Leu Cys Thr Leu Ala Leu Trp Ala  
 1 5 10  
 aag tca gtc agt gct ggc tcc agc ttc ctc agc ccc tcc cag aaa cca 95  
 55 Lys Ser Val Ser Ala Gly Ser Ser Phe Leu Ser Pro Ser Gln Lys Pro

15                      20                      25  
 cag gta aga cag ggt aaa ggg aag ccc cct cga gtt ggt cgg cga gac 143  
 5 Gln Val Arg Gln Gly Lys Gly Lys Pro Pro Arg Val Gly Arg Arg Asp  
     30                      35                      40  
 att gag agc ttt gct gag ctg ttt gag ggt ccc ctt cac cag gaa gac 191  
 10 Ile Glu Ser Phe Ala Glu Leu Phe Glu Gly Pro Leu His Gln Glu Asp  
     45                      50                      55                      60  
 aaa cac aat acg atc aag gct cct ttt gag atg ggc atc acc atg agt 239  
 15 Lys His Asn Thr Ile Lys Ala Pro Phe Glu Met Gly Ile Thr Met Ser  
     65                      70                      75  
 gag gag gag ttc cag gag tatggtgccg tgctgcagaa gatcctgcag 287  
 Glu Glu Glu Phe Gln Glu  
 20                      80  
 gacgtcctgg gagacactgc cactgcagaa tgatcacaac ttggcataga cacggaatac 347  
 aaagaacctc cattccctgt tctccaactt tcotcttctca acttgtctta tacccaatgt 407  
 25 actgtgtgaa catcgtttga attgtaaaag atgaataaaa taaccgcggc cgcta 462  
  
 <210> 39  
 <211> 453  
 30 <212> DNA  
 <220>  
 <221> CDS  
 35 <222> (12)... (308)  
 <213> Oncorhynchus mykiss  
 <223> Base sequence of cDNA coding prepro-form of rainbow trout  
 endogenous peptides (20 amino acids) of growth hormone  
 40 secretagogue  
 <400> 39  
 tcacaggtct c atg ata ctg atg ctg tgt act ctg gct ctg tgg gcc 47  
 45 Met Ile Leu Met Leu Cys Thr Leu Ala Leu Trp Ala  
     1                      5                      10  
 aag tca gtc agt gct ggc tcc agc ttc ctc agc ccc tcc cag aaa cca 95  
 50 Lys Ser Val Ser Ala Gly Ser Ser Phe Leu Ser Pro Ser Gln Lys Pro  
     15                      20                      25  
 cag ggt aaa ggg aag ccc cct cga gtt ggt cgg cga gac att gag agc 143  
 55 Gln Gly Lys Gly Lys Pro Pro Arg Val Gly Arg Arg Asp Ile Glu Ser



25

## Claims

- 35 1. A peptide-type compound, wherein in a peptide having the activity of increasing the intracellular calcium ion concentration, at least one amino acid is replaced by a modified amino acid and/or a non-amino acid compound, or a pharmaceutically acceptable salt thereof.
- 40 2. The peptide-type compound according to claim 1, which comprises (a) an amino acid sequence set forth in SEQ ID NO: 2 or (b) an amino acid-sequence having any one of amino acid sequences selected from the group consisting of
- 45 (1) amino acid sequence of amino acids 1 to 4,  
(2) amino acid sequence of amino acids 1 to 5,  
(3) amino acid sequence of amino acids 1 to 6,  
(4) amino acid sequence of amino acids 1 to 7,  
(5) amino acid sequence of amino acids 1 to 8,  
(6) amino acid sequence of amino acids 1 to 9, and  
50 (7) amino acid sequence of amino acids 1 to 10
- from the amino-terminal in the sequence (a) and at least one amino acid deleted, replaced and/or added in a part outside said amino acid sequences, or a pharmaceutically acceptable salt thereof.
- 55 3. The peptide-type compound according to claim 2, which comprises one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 3, 4, 5, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, 22 and 23, or a pharmaceutically acceptable salt thereof.
4. The peptide-type compound according to claim 2, which comprises one amino acid sequence selected from the

group consisting of amino acid sequences set forth in SEQ ID NOS: 25, 26, 29, 30, 31, 32, 34 and 35, or a pharmaceutically acceptable salt thereof.

- 5 5. A peptide-type compound, wherein in a peptide having the activity of increasing the intracellular calcium ion concentration and the activity of inducing secretion of growth hormone, (a) constitutional amino acids are modified or not modified and (b) at least one amino acid is replaced or not replaced by a non-amino acid compound, or a pharmaceutically acceptable salt thereof.
- 10 6. The peptide-type compound according to claim 1 or 5 which comprises amino acid sequences set forth in SEQ ID NOS: 27, 28 and 33, or a pharmaceutically acceptable salt thereof.
7. The peptide-type compound according to claim 5, which comprises (a) an amino acid sequence set forth in SEQ ID NO: 2 or (b) an amino acid sequence having any one of amino acid sequences selected from the group consisting of
  - 15 (1) amino acid sequence of amino acids 1 to 4,
  - (2) amino acid sequence of amino acids 1 to 5,
  - (3) amino acid sequence of amino acids 1 to 6,
  - (4) amino acid sequence of amino acids 1 to 7,
  - 20 (5) amino acid sequence of amino acids 1 to 8,
  - (6) amino acid sequence of amino acids 1 to 9, and
  - (7) amino acid sequence of amino acids 1 to 10

from the amino-terminal in the sequence (a) and at least one amino acid deleted, replaced and/or added in a part outside said amino acid sequences, or a pharmaceutically acceptable salt thereof.

8. The peptide-type compound according to claim 7, which comprises one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 3, 4, 5, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, 22 and 23, or a pharmaceutically acceptable salt thereof.
- 30 9. The peptide-type compound according to claim 7, which comprises one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 25, 26, 29, 30, 31, 32, 34 and 35, or a pharmaceutically acceptable salt thereof.
- 35 10. The peptide-type compound according to claim 1 or 5, whose amino-terminal amino acids 1 to 4 are represented by formula:



40 wherein the symbol A is either an amino acid or a non-amino acid compound, or is missing, and the symbol B is either an amino acid or a non-amino acid compound, or is missing, provided that the length of the A + B molecular chain is a dipeptide length, and the symbol C or the symbol D may be the same or different and represents (a) a modified amino acid, (b) an amino acid having a hydrophobic residue, or (c) an amino acid having a basic side chain, or a pharmaceutically acceptable salt thereof.

11. The peptide-type compound according to claim 10, wherein the symbol C is a modified amino acid in which (a) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid via or not via an alkylene group containing one or more carbon atoms and via an ester, ether, thioether, amide or disulfide linkage, or (b) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid, and the symbol D is an amino acid having a hydrophobic residue, or a pharmaceutically acceptable salt thereof.
- 50 12. A peptide-type compound, wherein in one amino acid sequence selected from the group consisting of amino acid sequences set forth in SEQ ID NOS: 2, 3, 9, 10, 11, 16, 17, 22, 25, 26, 27, 28, 29, 30 and 31, an amino acid sequence of amino-terminal amino acids 1 to 4 is replaced by the structure of the peptide-type compound described in claim 10 or 11, or a pharmaceutically acceptable salt thereof.
- 55

13. The peptide-type compound according to claim 1, 2, 3, 5, 7 or 8, wherein the modified amino acid is an amino acid at the 3rd position from the amino-terminal, or a pharmaceutically acceptable salt thereof.
- 5 14. The peptide-type compound according to claim 13, wherein the amino acid in the modified amino acid is serine or cysteine, or a pharmaceutically acceptable salt thereof.
- 10 15. The peptide-type compound according to claim 1, 2, 3, 5, 7 or 8, which comprises a modified amino acid in which (a) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid via or not via an alkylene group containing one or more carbon atoms and via an ester, ether, thioester, thioether, amide or carbamide linkage, or (b) H or a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom of the amino acid, or a pharmaceutically acceptable salt thereof.
- 15 16. The peptide-type compound according to claim 1, 2, 4, 5, 6, 7, 9, 10 or 12, wherein the modified amino acid is an amino acid in which (a) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon atom via or not via an alkylene group containing one or more carbon atoms and via an ester, ether, thioester, thioether, disulfide, amide, carbamide or thiocarbamide linkage, or (b) a saturated or unsaturated alkyl chain containing one or more carbon atoms was introduced at the  $\alpha$  carbon, or a pharmaceutically acceptable salt thereof.
- 20 17. The peptide-type compound according to claim 1, 2, 3, 5, 7 or 8, which comprises a modified amino acid modified with an ester linkage, or a pharmaceutically acceptable salt thereof.
- 25 18. The peptide-type compound according to claim 1, 2, 4, 5, 6, 7, 9, 10, 11 or 12, which comprises a modified amino acid modified by conversion of a functional group in a side chain of said amino acid into an ester linkage, or a pharmaceutically acceptable salt thereof.
- 30 19. The peptide-type compound according to claim 17, which comprises an amino acid having a fatty acid bound via an ester linkage to a side-chain hydroxyl group of said amino acid, or a pharmaceutically acceptable salt thereof.
- 35 20. The peptide-type compound according to claim 18, which comprises an amino acid having a fatty acid bound via an ester linkage to a side-chain hydroxyl group of said amino acid or via a thioester linkage to a side-chain mercapto group of said amino acid, or a pharmaceutically acceptable salt thereof.
- 40 21. The peptide-type compound according to claim 19, which comprises an amino acid to which a fatty acid containing 2 to 35 carbon atoms was bound, or a pharmaceutically acceptable salt thereof.
- 45 22. The peptide-type compound according to claim 20, wherein the fatty acid contains 2 to 35 carbon atoms, or a pharmaceutically acceptable salt thereof.
- 50 23. The peptide-type compound according to claim 21, which comprises an amino acid to which a fatty acid selected from the group consisting of fatty acids containing 2, 4, 6, 8, 10, 12, 14, 16 and 18 carbon atoms was bound, or a pharmaceutically acceptable salt thereof.
- 55 24. The peptide-type compound according to claim 22, wherein the fatty acid is a fatty acid selected from the group consisting of fatty acids containing 2, 4, 6, 8, 10, 12, 14, 16 and 18 carbon atoms, or a pharmaceutically acceptable salt thereof.
25. The peptide-type compound according to claim 23, wherein the bound fatty acid is octanoic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof.
26. The peptide-type compound according to claim 24, wherein the fatty acid is octanoic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof.
27. The peptide-type compound according to claim 23, wherein the bound fatty acid is decanoic acid, a monoene fatty acid thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof.
28. The peptide-type compound according to claim 24, wherein the fatty acid is decanoic acid, a monoene fatty acid

thereof or a polyene fatty acid thereof, or a pharmaceutically acceptable salt thereof.

29. A peptide-type compound comprising a basic amino acid bound to the carboxyl-terminal of a peptide-type compound described in claims 1 to 28.

30. The peptide-type compound according to claims 1, 2, 3, 5, 7, 8, 13, 14, 15, 17, 19, 21, 23, 25 and 27, wherein the amino-terminal is modified with a saturated or unsaturated alkyl or acyl group containing one or more carbon atoms, and/or a hydroxyl group of the carboxyl-terminal carboxyl group is OZ or NR<sub>2</sub>R<sub>3</sub> wherein Z is a pharmaceutically acceptable cation or a lower branched or linear alkyl group, and R<sub>2</sub> and R<sub>3</sub> are the same or different and represent H or a lower branched or linear alkyl group.

31. The peptide-type compound according to claims 1, 2, 4, 5, 6, 7, 9, 10, 11, 12, 16, 18, 20, 22, 24, 26, 28 and 29, wherein the amino-terminal amino group is modified by introduction of a saturated or unsaturated alkyl or acyl group containing one or more carbon atoms, and/or a hydroxyl group of the carboxyl-terminal carboxyl group is OZ or NR<sub>2</sub>R<sub>3</sub> wherein Z is a pharmaceutically acceptable cation or a lower branched or linear alkyl group, and R<sub>2</sub> and R<sub>3</sub> are the same or different and represent H or a lower branched or linear alkyl group.

32. A peptide-type compound comprising a basic group introduced into a carboxyl-terminal amide derivative of a peptide-type compound described in claim 30 or 31.

33. A pharmaceutical composition comprising a peptide-type compound described in claims 1 to 32 or a pharmaceutically acceptable salt thereof as an active ingredient.

34. A pharmaceutical composition for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises a peptide-type compound described in claims 1 to 32 or a pharmaceutically acceptable salt thereof as an active ingredient.

35. A pharmaceutical composition for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises an agent for treating diseases not attributable to a defect or decrease in growth hormone and a peptide-type compound described in claims 1 to 32 or a pharmaceutically acceptable salt thereof.

36. A pharmaceutical composition according to claims 33 to 35, which is applied to animals other than human beings.

37. A method for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises administering a pharmaceutical composition comprising a peptide-type compound described in claims 1 to 32 or a pharmaceutically acceptable salt thereof as an active ingredient.

38. A method for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises administering an agent for treating diseases not attributable to a defect or decrease in growth hormone and a peptide-type compound described in claims 1 to 32 or a pharmaceutically acceptable salt thereof.

39. The treatment method according to claims 37 to 38, which is applied to animals other than human beings.

40. A DNA coding for an amino acid sequence of a peptide-type compound described in claims 1 to 32, which comprises a nucleotide sequence coding for a peptide containing an amino acid sequence recognizing at least one modifiable amino acid in the amino acid sequence encoded by said DNA.

41. The DNA according to claim 40, wherein the nucleotide sequence is one nucleotide sequence selected from the group consisting of nucleotide sequences set forth in SEQ ID NOS: 6, 7, 14, 15, 20, 21, 24, 36, 37, 38 and 39.

42. The DNA according to claim 40, wherein the nucleotide sequence is an amino acid-coding nucleotide sequence in one nucleotide sequence selected from the group consisting of nucleotide sequences set forth in SEQ ID NOS: 6, 7, 14, 15, 20, 21, 24, 36, 37, 38 and 39.

43. A vector comprising a DNA described in claims 40 to 42.

44. Cells comprising the vector described in claim 43.

45. Cells comprising a DNA described in claims 40 to 42, wherein a peptide-type compound having an amino acid sequence encoded by said DNA can be produced as a peptide-type compound having at least one amino acid modified in said amino acid sequence.
- 5 46. An antibody against a peptide-type compound described in claims 1 to 32.
47. A method for assaying a peptide-type compound described in claims 1 to 32, which comprises using the antibody described in claim 46 to detect the peptide-type compound described in claims 1 to 32.
- 10 48. A kit for detecting a peptide-type compound described in claims 1 to 32, which comprises using the antibody described in claim 46 to detect the peptide-type compound described in claims 1 to 32.
49. A method for producing a peptide-type compound described in claims 1 to 32 by genetic recombination technology, which comprises transforming a vector containing a DNA described in claims 40 to 42 into host cells capable of  
15 modifying a side chain of at least one amino acid in said peptide, then culturing the resulting transformed cells and recovering the desired peptide-type compound from the culture.
50. A method for producing a peptide-type compound described in claims 1 to 32 by genetic recombination technology, which comprises transforming a vector containing a DNA described in claims 40 to 42 into host cells, then culturing  
20 the resulting transformed cells and recovering the desired peptide-type compound from the culture, followed by chemically modifying an arbitrary amino acid thereof.
51. A method for producing a peptide-type compound described in claims 19 to 28 by genetic recombination technology, which comprises using cells having the activity of binding a fatty acid via an ester linkage to a side-chain  
25 hydroxyl group of an amino acid or via a thioester linkage to a side-chain mercapto group of an amino acid in the peptide-type compound.
52. A method for producing a peptide-type compound described in claims 19 to 28, which comprises using cells having the serine acylation activity of binding a fatty acid via an ester linkage to a side-chain hydroxyl group of serine in  
30 the amino acid sequence set forth in SEQ ID NO: 8.
53. A method for producing a peptide-type compound described in claims 19 to 28, which comprises using cells having the acylation activity of binding a fatty acid via an ester linkage to a side-chain hydroxyl group of threonine in the  
35 amino acid sequence set forth in SEQ ID NO: 28.
54. A pharmaceutical composition for gene therapy for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding for an amino acid sequence of a  
40 peptide-type compound described in claims 1 to 32 into cells in a living body and expressing a peptide with at least one modified amino acid, the peptide having the activity of increasing the intracellular calcium ion concentration.
55. A method for treatment of diseases attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding for an amino acid sequence of a peptide-type compound described  
45 in claims 1 to 32 into cells in a living body enabling a peptide having an amino acid sequence encoded by said DNA to be produced as a peptide having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence, whereby a peptide having the activity of inducing growth hormone is expressed.
56. A pharmaceutical composition for gene therapy for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding for an amino acid sequence of a  
50 peptide-type compound described in claims 1 to 32 into cells in a living body and expressing a peptide with at least one modified amino acid, the peptide having the activity of increasing the intracellular calcium ion concentration.
57. A method for treatment of diseases not attributable to a defect or decrease in growth hormone, which comprises integrating a vector containing a DNA coding for an amino acid sequence of a peptide-type compound described  
55 in claims 1 to 32 into cells in a living body enabling a peptide having an amino acid sequence encoded by said DNA to be produced as a peptide having an amino acid sequence recognizing at least one modifiable amino acid in said amino acid sequence, whereby a peptide having the activity of inducing growth hormone is expressed.

Figure

Fig. 1

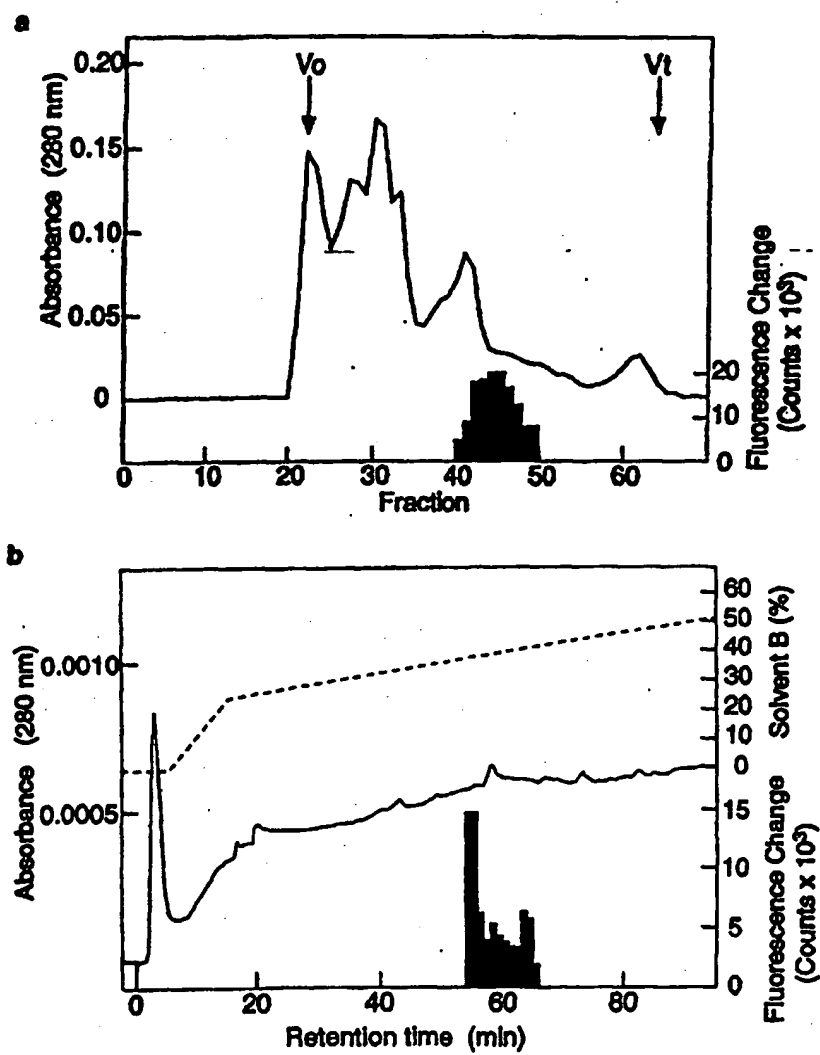


Fig. 2

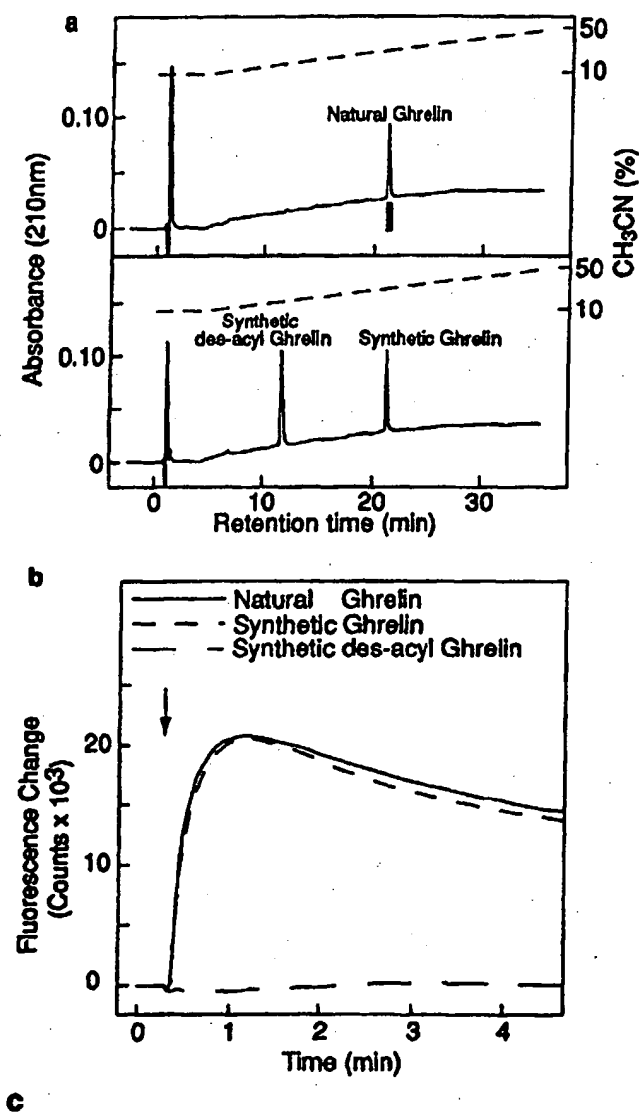


Fig. 3

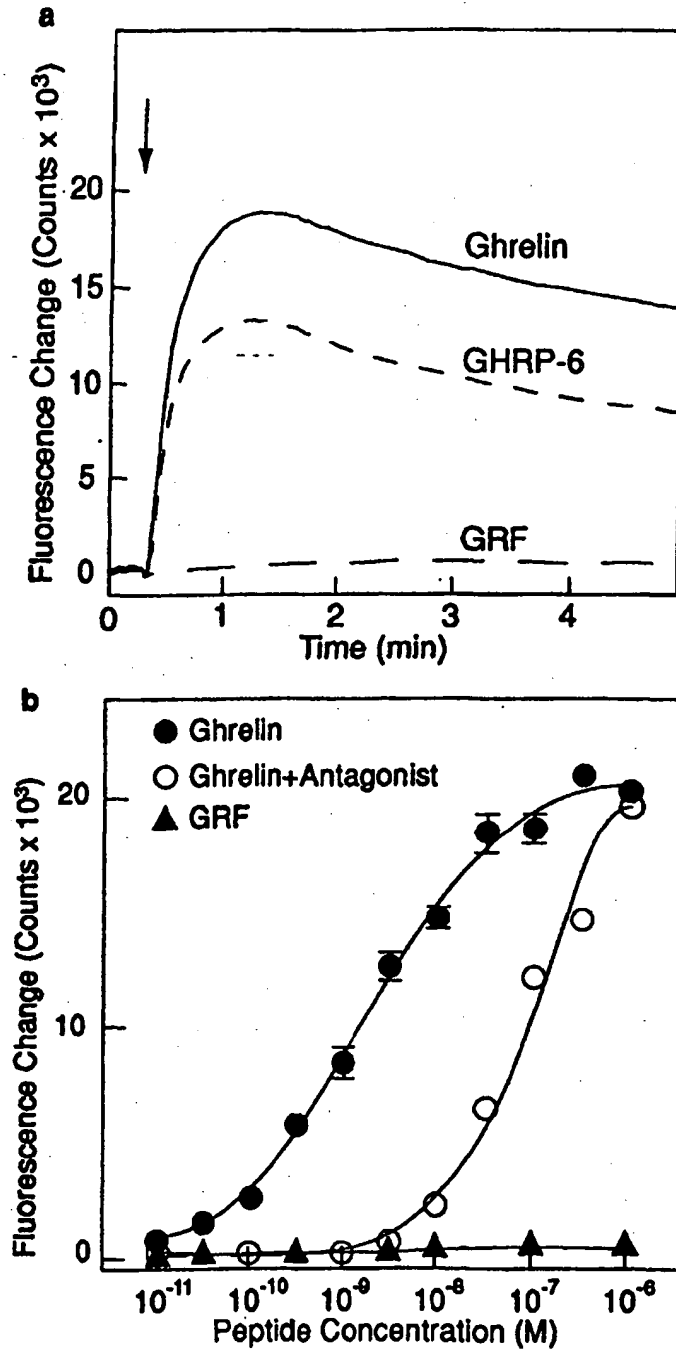




Fig. 4

a

Human	1	MPSPGTVCSLLLLGMLWDLAMAGSSFLSP	30
Rat	1	MVSSATICSLLLLSMLWMDMAMAGSSFLSP	30
Human	31	EHORVOORKESKKPPAKLOPRALAGWLRPE	60
Rat	31	EHOKAQORKESKKPPAKLOPRALEGWLHPE	60
Human	61	DGGQAEGAEDELEVRFNAPFDVGIKLSGVQ	90
Rat	61	DRGQAEAEAELEIRFNAPFDVGIKLSGAQ	90
Human	91	YQQHSQALGKFLQDILWEEAKEAPADK	117
Rat	91	YQQHGRALGKFLQDILWEEVKEAPANK	117

b

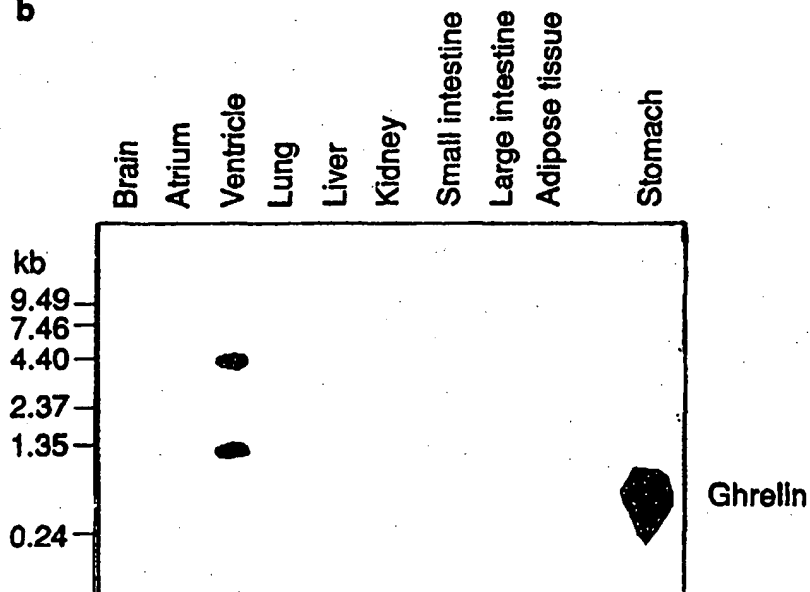


Fig. 5

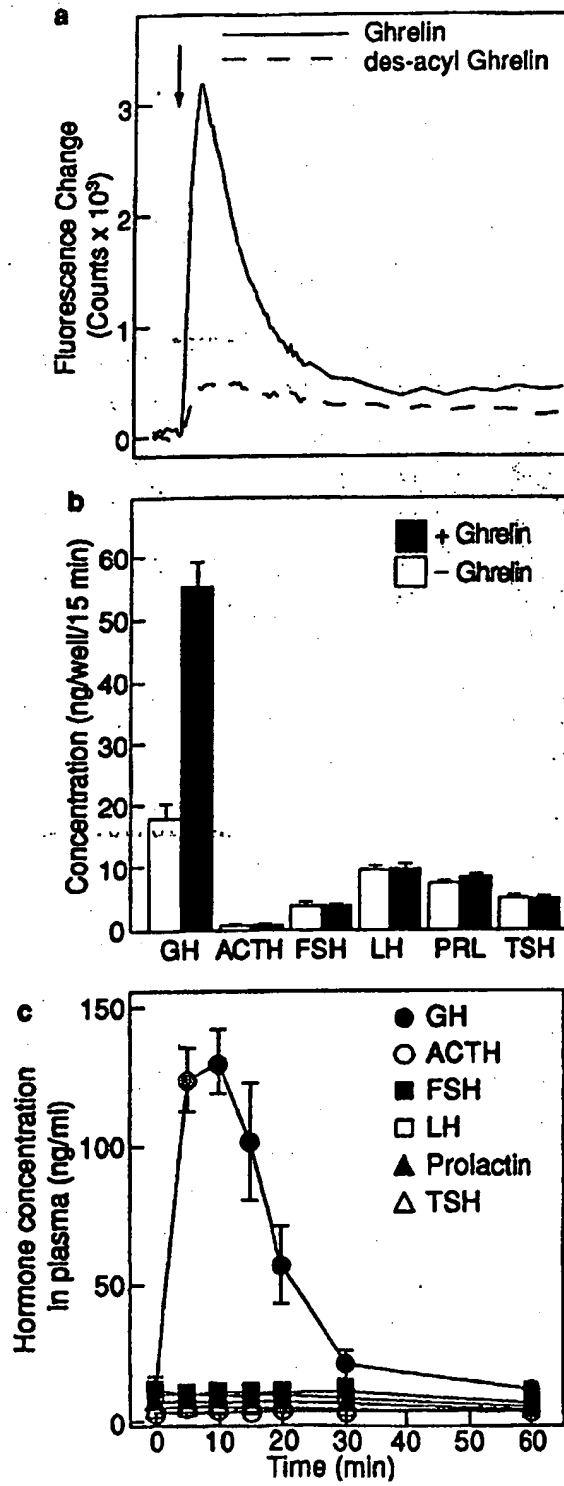


Fig. 6

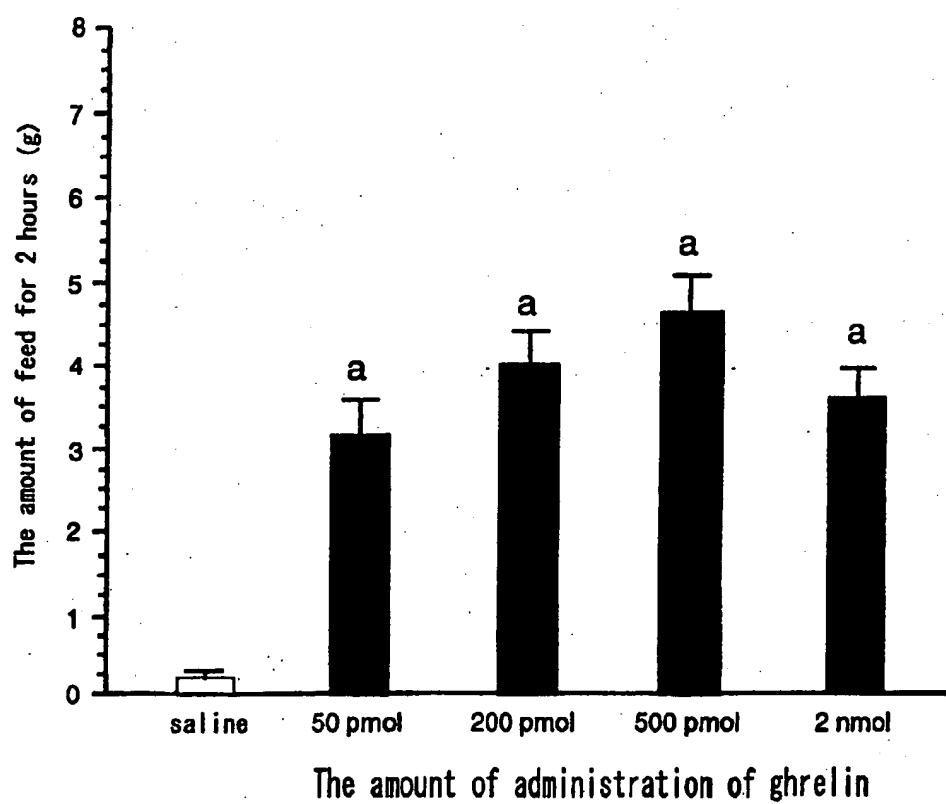
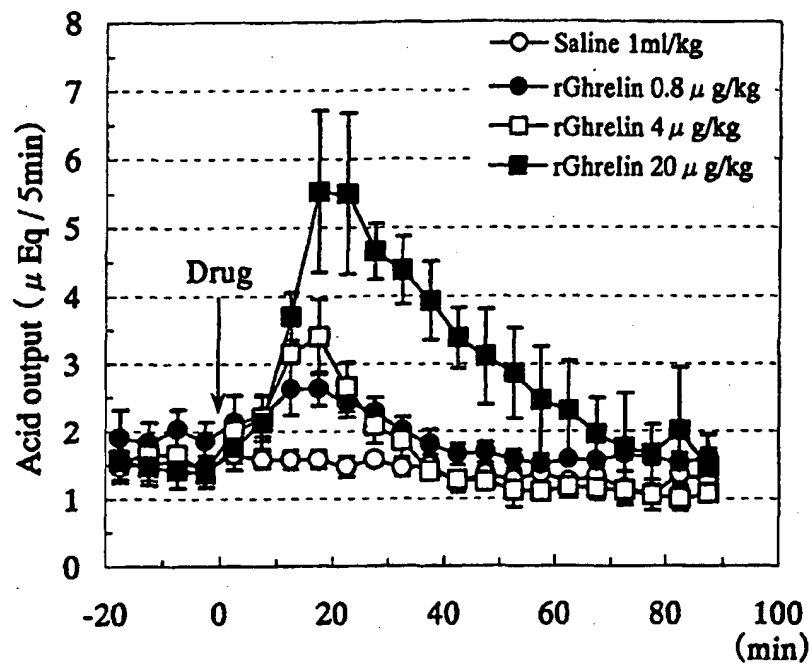


Fig. 7

A



B

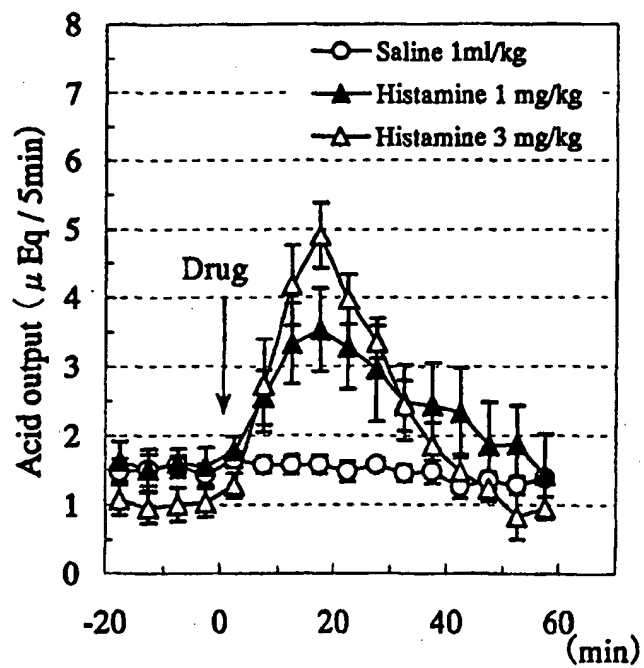


Fig. 8

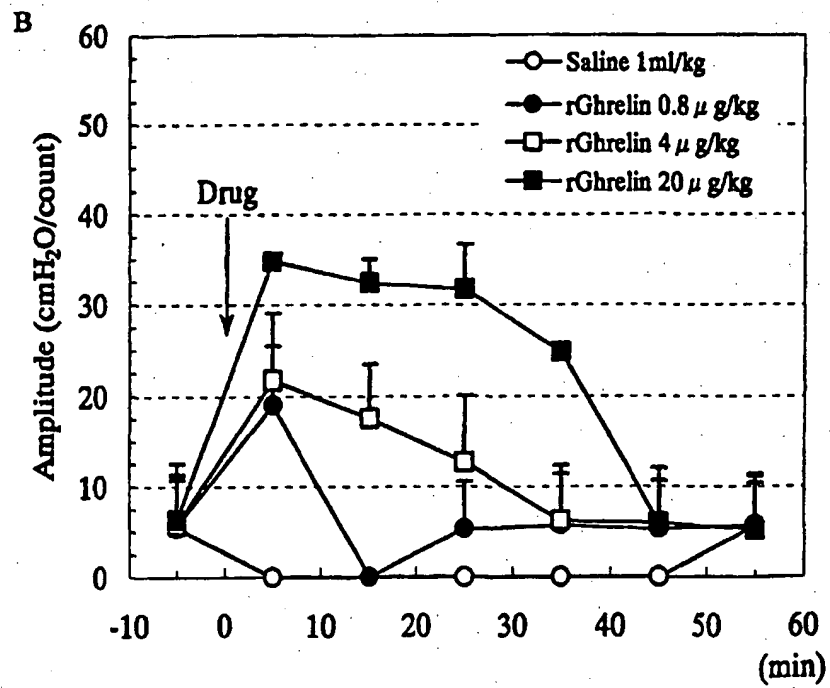
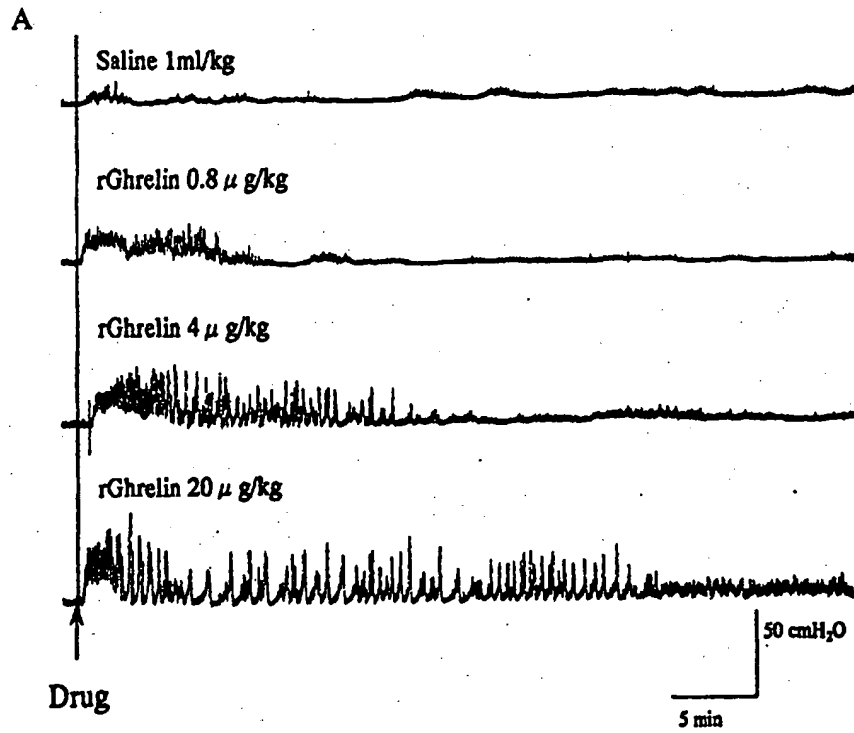
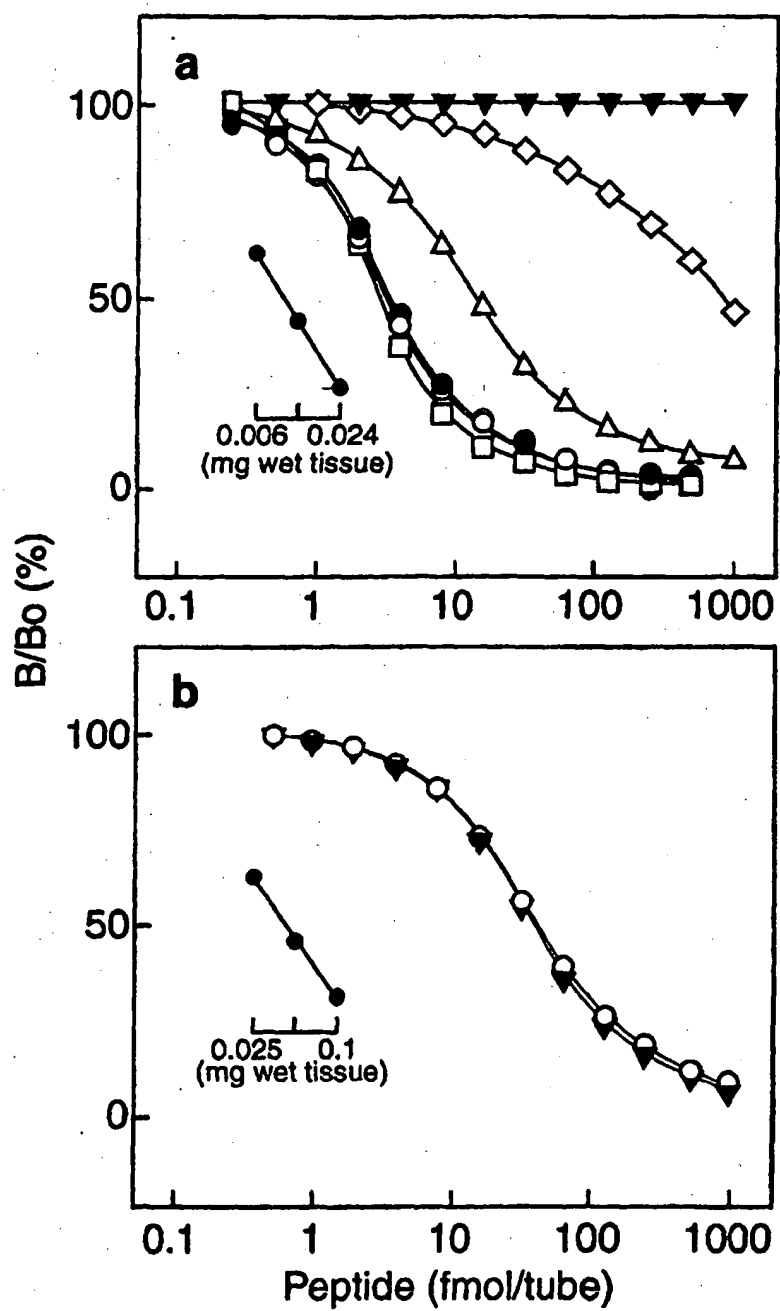


Fig. 9



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/04907

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int.Cl <sup>7</sup> C07K14/47, C12N15/12, C12N1/21, C12P21/02, C07K16/18, A61K38/18, A61P5/06, A61P19/08, A61K45/00, A61K48/00, G01N33/53 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) Int.Cl <sup>7</sup> C07K14/47, C12N15/12, C12N1/21, C12P21/02, C07K16/18, A61K38/18, A61P5/06, A61P19/08, A61K45/00, A61K48/00, G01N33/53 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) SwissProt/PIR/GeneSeq, Genbank/EMBL/DBJ/GeneSeq, CA (STN), REGISTRY (STN), WPI (DIALOG), BIOSIS (DIALOG)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, 98/42840, A1 (ZYMOGENETICS, INC.), 01 October, 1998 (01.10.98), p.19, pp.54-58 & AU, 9865769, A & NO, 9904614, A & EP, 975760, A1 & BR, 9808059, A & CN, 1254375, A	1-32, 40-53
X	BLUET-PAJOT, M-T. et al., "Hypothalamic and hypophyseal regulation of growth hormone secretion", Cellular and Molecular Neurobiology (1998), Vol.18, No.1 pp.101-104, p.109	1, 5, 33-36, 39, 54, 56
P, X	KOJIMA, M. et al., Ghrelin is a growth-hormone-releasing acylated peptide from stomach", NATURE (Dec.1999), Vol.402, No.9, pp.656-660	1-36, 39-54, 56
P, X	HOSODA, H. et al., "Purification and characterization of rat des-Gln <sup>14</sup> -Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor", J. Biol. Chem. (MAY, 2000), Vol.275, No.29, pp.21995-22000	1-36, 39-54, 56
P, X	WO, 99/63088, A2 (GENENTECH, INC.),	1-32, 40-53
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 17 October, 2000 (17.10.00)		Date of mailing of the international search report 24 October, 2000 (24.10.00)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/04907

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	09 December, 1999 (09.12.99), & AU, 9943286	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/04907

**Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 37-39,55,57  
because they relate to subject matter not required to be searched by this Authority, namely:  
Claims 37, 38, 55 and 57 pertain to methods for treatment of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required to search.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

## EXHIBIT 4

# Crystal structure of the outer membrane protease OmpT from *Escherichia coli* suggests a novel catalytic site

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<sup>†</sup>Deceased

OmpT from *Escherichia coli* belongs to a family of highly homologous outer membrane proteases, known as omptins, which are implicated in the virulence of several pathogenic Gram-negative bacteria. Here we present the crystal structure of OmpT, which shows a 10-stranded antiparallel  $\beta$ -barrel that protrudes far from the lipid bilayer into the extracellular space. We identified a putative binding site for lipopolysaccharide, a molecule that is essential for OmpT activity. The proteolytic site is located in a groove at the extracellular top of the vase-shaped  $\beta$ -barrel. Based on the constellation of active site residues, we propose a novel proteolytic mechanism, involving a His–Asp dyad and an Asp–Asp couple that activate a putative nucleophilic water molecule. The active site is fully conserved within the omptin family. Therefore, the structure described here provides a sound basis for the design of drugs against omptin-mediated bacterial pathogenesis. Coordinates are in the Protein Data Bank (accession No. 1I78)

**Keywords:** His–Asp dyad/lipopolysaccharide/OmpT/omptin/protease

## Introduction

Omptins are outer membrane proteases found in several Gram-negative bacteria and include OmpT of *Escherichia coli* (Sugimura and Nishihara, 1988), PgtE of *Salmonella typhimurium* (mature part 49% identical to OmpT) (Yu and Hong, 1986), Pla of *Yersinia pestis* (50% identical) (Sodeinde and Goguen, 1989), SopA of *Shigella flexneri* (60% identical) (Egile *et al.*, 1997) and OmpP of *E.coli* (72% identical) (Kaufmann *et al.*, 1994). Several studies have implicated the omptin family in the pathogenicity of these bacteria (Stathopoulos, 1998). The presence of the *ompT* gene in clinical isolates of *E.coli* has been associated with complicated urinary tract disease (Webb and Lundigran, 1996), a notion supported by the observation that OmpT cleaves protamine, a highly basic antimicrobial

peptide that is excreted by epithelial cells of the urinary tract (Stumpe *et al.*, 1998). Inactivation of the gene encoding Pla in *Y.pestis*, the causative agent of plague, increased the median lethal dose of the bacterium for mice by 10<sup>6</sup>-fold (Sodeinde *et al.*, 1992). The role of Pla in pathogenicity might be related to its ability to activate plasminogen, a feature shared with OmpT (Lundigran and Webb, 1992). SopA from *S.flexneri*, the causative agent of bacillary dysentery, cleaves the endogenous autotransporter IcsA that has an essential role in the formation of actin tails in host cells, and therefore SopA might be involved in actin-based motility inside infected cells (Egile *et al.*, 1997; Shere *et al.*, 1997). Thus, the proteolytic activity of the omptins is probably involved in a variety of ways in the pathogenicity of these bacteria, ranging from bacterial defence and plasmin-mediated tissue infiltration to motility inside infected cells.

OmpT is biochemically the best characterized member of the omptins. It preferentially cleaves substrates between two consecutive basic amino acids (Dekker *et al.*, 2001). The protease displays optimal activity at alkaline pH and it is dependent on lipopolysaccharide (LPS), showing no detectable enzymatic activity towards peptide substrates in the absence of LPS (Kramer *et al.*, 2000b). Hydrolysis of a fluorogenic peptide substrate by OmpT was characterized by a specificity constant  $k_{cat}/K_m$  of 10<sup>8</sup> s<sup>-1</sup> M<sup>-1</sup>, indicating a cleavage efficiency comparable with that of water-soluble proteases such as chymotrypsin (Kramer *et al.*, 2000b). The enzyme does not contain any conserved active site sequence found in other known protease families. In addition, commonly used serine protease inhibitors have little or no effect on the activity of OmpT (Sugimura and Nishihara, 1988). However, because some serine protease inhibitors weakly affect OmpT activity, the omptins have been classified as novel serine proteases (family S18) (Rawlings and Barrett, 1994). Site-directed mutagenesis studies appeared to support this classification, since Ser99 and His212 have been found to be important for the activity of OmpT (Kramer *et al.*, 2000a).

Here we describe the first structure of an integral outer membrane protease, OmpT from *E.coli*, at 2.6 Å resolution. The crystallized protein contained three mutations to prevent autoproteolytic degradation. Based on the structure, we identified a putative LPS-binding site and provide support for the omptins constituting a novel class of proteases.

## Results and discussion

### Structure determination

The OmpT structure was determined with the single-wavelength anomalous diffraction (SAD) phasing method using a seleno-methionine (Se-Met)-containing OmpT crystal, which diffracted to 3.1 Å resolution. The final

Table I. Summary of data and refinement statistics

	Native	Se-Met
Data set statistics		
resolution limits (outer shell) (Å)	20–2.6 (2.69–2.6)	40–3.1 (3.21–3.1)
space group	$P3_121$	$P3_121$
unit cell parameters (Å, °)	$a = 98.39$ $b = 98.39$ $c = 165.70$ $\alpha = 90, \beta = 90, \gamma = 120$	$a = 97.79$ $b = 97.79$ $c = 165.86$ $\alpha = 90, \beta = 90, \gamma = 120$
mosaicity (°)	0.1	0.8
oscillation range (°)	0.2	0.5
total oscillation for data set (°)	25	100
total no. of reflections (outer shell)	43 471 (4352)	107 336 (10 902)
no. of unique reflections (outer shell) <sup>a</sup>	27 400 (2766)	17 252 (1705)
redundancy	1.59 (1.57)	6.22 (6.39)
$R_{\text{merge}}$ (%) (outer shell) <sup>b</sup>	7.4 (26.3)	9.7 (39.5)
completeness (%) (outer shell)	94.3 (97.7)	99.8 (100)
$I/\sigma(I)$ (outer shell)	8.82 (2.54)	15.48 (4.56)
Refinement statistics		
resolution range (Å)	20–2.6	
total no. of non-hydrogen atoms	4734	
no. of water molecules	29	
no. of $\beta$ -OG molecules	4	
$R_{\text{work}}$ (%)	23.7	
$R_{\text{free}}$ (%)	28.0	
r.m.s.d. bond lengths (Å)	0.007	
r.m.s.d. bond angles (°)	1.35	
average $B$ -factor (all protein atoms) (Å <sup>2</sup> )	52.6	

<sup>a</sup>For the Se-Met data set, the Friedel mates have been counted separately.

<sup>b</sup> $R_{\text{merge}} = \sum(I - \langle I \rangle) / \sum(I)$ .

structure was refined against data to 2.6 Å resolution using a crystal of OmpT containing natural methionines. The enzyme contained the mutations S99A, G216K and K217G, to abolish autoproteolytic activity. Additionally, crystals of a S99A mutant were obtained that diffracted to a lower resolution of 3.2 Å, yielding a map indicating an identical structure (unpublished results). OmpT was crystallized using 1% (w/v) octyl- $\beta$ -D-glucopyranoside (OG), 30% (v/v) 2-methyl-2,4-pentanediol (MPD) and 0.3 M sodium citrate pH 5.5. It crystallized in the space group  $P3_121$  with unit cell dimensions  $a = b = 97.8$  Å and  $c = 165.3$  Å. Two OmpT molecules are present in the asymmetric unit that show a high degree of structural similarity, with a root mean square deviation (r.m.s.d.) of 0.50 Å for main-chain atoms. The refined model consists of 584 amino acid residues, four OG and 29 water molecules, and has a crystallographic  $R$ -factor of 23.7% and an  $R_{\text{free}}$  of 28.0% for data in the 20–2.6 Å resolution range. Table I summarizes the statistics of the crystallographic data and the refinement. Coordinates and structure factors have been deposited in the Protein Data Bank with accession No. 1I178.

### Overall structure

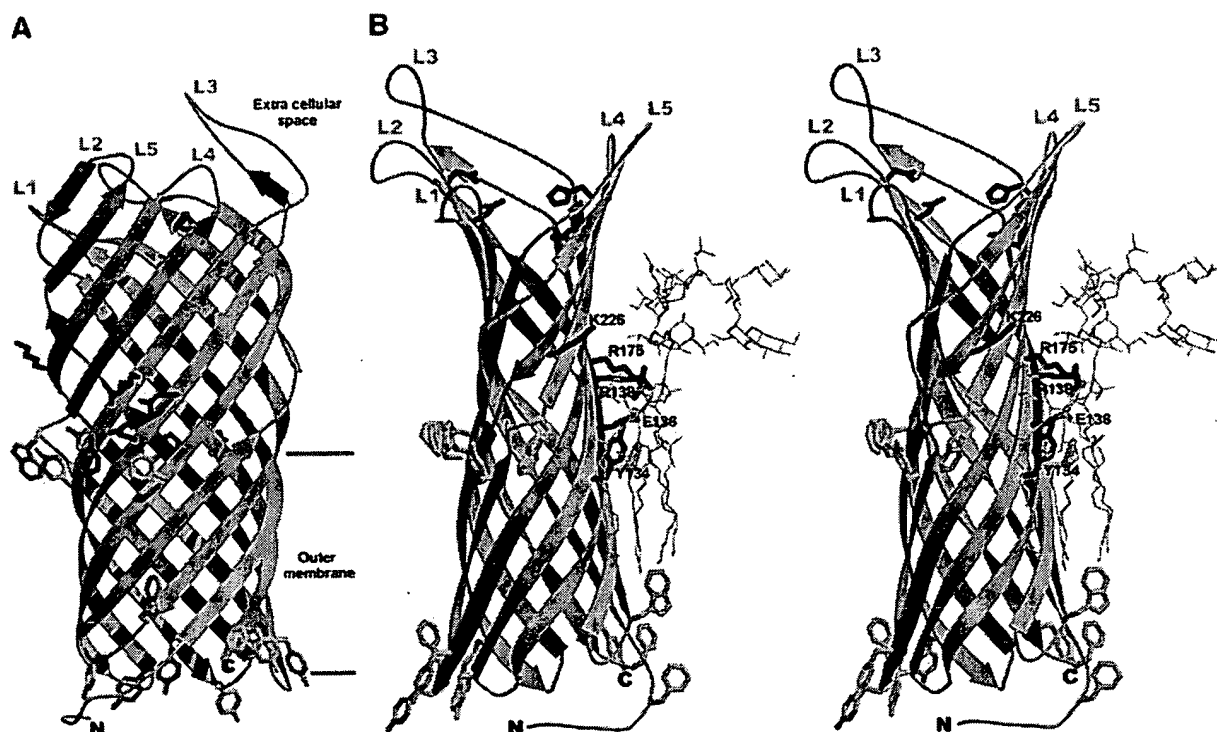
The overall structure of OmpT consists of an exceptionally long 10-stranded vase-shaped antiparallel  $\beta$ -barrel of ~70 Å in its longest dimension (Figure 1). The number, as well as the approximate position of the 10  $\beta$ -strands, was predicted correctly by Kramer *et al.* (2000b). The  $\beta$ -strands have an average length of 23 residues, have a tilt angle of 37–45° with respect to the barrel axis and have a shear number of 12 (Figure 2). Like other outer membrane

proteins (Weiss *et al.*, 1990; Cowan *et al.*, 1992; Kreusch *et al.*, 1994; Schirmer *et al.*, 1995; Ferguson *et al.*, 1998; Locher *et al.*, 1998; Pautsch and Schulz, 1998; Buchanan *et al.*, 1999; Snijder *et al.*, 1999; Vogt and Schulz, 1999; Koronakis *et al.*, 2000), OmpT contains short turns at the periplasmic side of the barrel, long, more mobile loops (Figure 3) at the extracellular part and a hydrophobic band of 25 Å in height flanked by aromatic residues that determine the position of the molecule in the membrane. On the extracellular side, OmpT protrudes ~40 Å from the lipid bilayer with the outermost loops located just above the rim of the LPS core region. Near the top of OmpT, the  $\beta$ -barrel has a circular diameter of ~32 Å, whereas in the central part the molecular cross-section is elliptical with dimensions of ~13 × ~26 Å, determined using the  $C_{\alpha}$  positions.

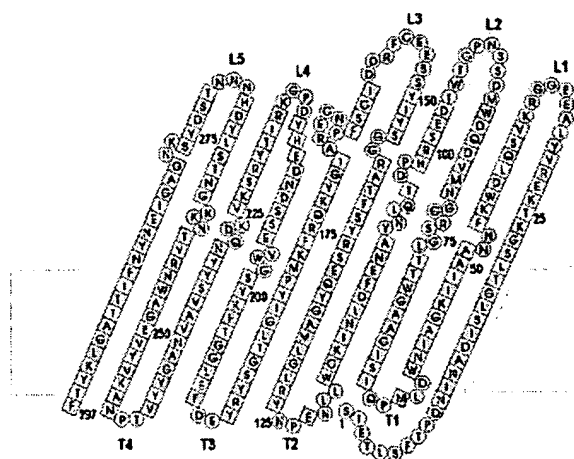
Within the membrane region, the barrel is highly regular, hollow and positively charged on the inside (Figure 4). Above the membrane, the barrel is constricted and distorted in its hydrogen bonding pattern; seven side chains, i.e. Ser22, Thr73, Arg77, Gln228, Asn258, Lys259 and Lys260, form hydrogen bonds to main chain atoms in the  $\beta$ -barrel. This constricted area, consisting of a few layers of predominantly conserved aromatic residues, forms the floor of a negatively charged groove that harbours the active site (discussed below).

### Putative LPS-binding site

*In vitro*, OmpT displays enzymatic activity only in the presence of LPS (Kramer *et al.*, 2000b). It is as yet unclear how LPS contributes to OmpT activity, but preliminary experiments suggest that one LPS molecule per OmpT is



**Fig. 1.** Overall structure of OmpT. (A) Ribbon representation of OmpT. The extracellular space is located at the top of the figure, and the periplasmic space is at the bottom. Extracellular loops are labelled L1–L5. The position of the membrane bilayer is delineated by horizontal lines. Aromatic residues that are located at the boundary of the hydrophobic and hydrophilic area on the molecular surface are coloured yellow. The proposed catalytic residues are depicted in red, and the purple-coloured residues show the putative LPS-binding site. (B) Stereo representation of a modelled LPS molecule at the putative binding site. The orientation of the OmpT molecule is rotated 90° along the barrel axis, with respect to (A). The OmpT–LPS model was obtained by superimposing the putative LPS-binding site of OmpT onto the LPS-binding site of FhuA (Ferguson *et al.*, 2000). LPS (from the FhuA structure) is shown by thin grey lines. The putative LPS-binding residues in purple are labelled. This figure, and Figures 5 and 7 were prepared using Bobscript (Esnouf, 1997) and Raster3D (Merritt and Bacon, 1997).



**Fig. 2.** Topology model of the OmpT  $\beta$ -barrel. Amino acid residues are given in one-letter code. Squares represent residues that are present in the  $\beta$ -strands. Side chains of amino acids that are shaded grey point to the outside of the barrel. Extracellular loops are labelled L1–L5 and periplasmic turns are labelled T1–T4. Every 25th residue is marked with the corresponding residue number. The grey area indicates the approximate position of the outer membrane.

sufficient for activity (unpublished results). Based on the structure of outer membrane protein FhuA from *E. coli* in complex with LPS, Ferguson *et al.* (1998, 2000) identified

a structural motif for LPS binding, consisting of four basic amino acids, which is conserved among pro- and eukaryotic LPS-binding proteins. Three of the four basic residues are found in a similar constellation in OmpT (Arg138, Arg175 and Lys226) (Figure 1B). The fourth conserved amino acid (Arg382 in FhuA) is lacking, possibly due to strong bending of OmpT at this position. Two additional amino acids that bind LPS in FhuA (Glu304 and Phe302) also have counterparts in OmpT (Glu136 and Tyr134). The five similar residues in FhuA and OmpT are at the same height in the barrel and have an r.m.s.d. of 1.1 Å for all atoms. Based on these similarities, we propose that the above-mentioned residues constitute an LPS-binding site in OmpT. A model of OmpT with an LPS molecule at the proposed binding site is shown in Figure 1B. The structure of OmpT enables us to test, using mutagenesis studies, whether these residues are indeed responsible for LPS binding and if they contribute to OmpT activity.

#### Active site

OmpT exerts its proteolytic activity in the extracellular medium (Kramer *et al.*, 2000b). The extracellular part of the molecule contains a large negatively charged groove, which is consistent with the substrate specificity of OmpT for two consecutive basic residues (Figure 4). All 18 residues in this proposed active site groove are fully

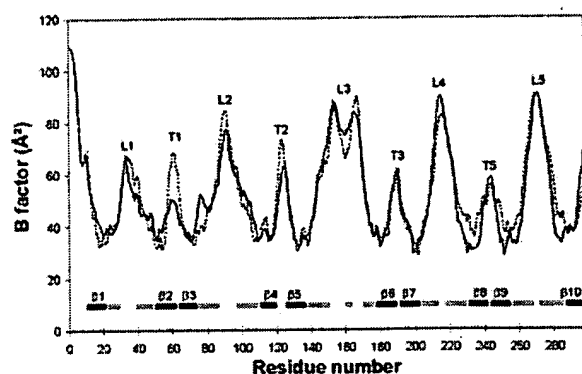


Fig. 3. B-factors of the C $\alpha$  atoms of the two OmpT molecules in the asymmetric unit, i.e. molecule A (solid line) and B (dashed line).  $\beta$ -strands are shown by bars that are black within the membrane-spanning region and grey outside this region. The positions of loops and turns are marked with L1–L5 and T1–T5, respectively.

conserved within the omptin family (Figures 5 and 6). Substitution of serines, histidines and acidic residues by alanines have resulted in ~10-fold reduced activity for Glu27, Asp97, Asp208 or His101, ~500-fold reduced activity for Ser99, and ~10 000-fold reduced activity for Asp83, Asp85, Asp210 or His212 (Kramer *et al.*, 2000a; R.A.Kramer, L.Vandeputte-Rutten, G.J.De Roon, P.Gros, N.Dekker and M.R.Egmond, submitted).

The structure contradicts the classification of OmpT as a serine protease, since the previously proposed catalytic residues Ser99 (alanine in the crystal structure) and His212 are too far apart, with a distance of 9 Å between C $\beta$  of Ser99 and N $\epsilon_2$  of His212. Furthermore, the single histidine located close to Ser99, His101, is only moderately important for catalysis. The four residues most important for activity form pairs, Asp83–Asp85 and His212–Asp210, which are located on opposite sides of the active site groove (Figure 7). The distance between the two couples is ~5 Å, determined by the closest distance between Asp83 and His212. The Asp83–Asp85 couple resembles the catalytic site of aspartic proteases. On the other hand, the His212–Asp210 couple resembles the His–Asp pair found in Ser–His–Asp triads of serine proteases. The observation that OmpT is active at high pH with a pK<sub>a</sub> of 6.2 (Kramer *et al.*, 2000b) suggests the involvement of an active site histidine. However, a putative nucleophilic residue is not present near the His212–Asp210 couple. We propose a mechanism in which a water molecule (invisible in the electron density map at the current resolution), positioned between Asp83 and His212, is activated by the His212–Asp210 dyad and subsequently performs the nucleophilic attack on the scissile peptide bond. A schematic model of the peptide substrate in the active site is given in Figure 7B.

OmpT is highly specific for lysine and arginine at position P1 (nomenclature according to Schechter and Berger, 1967) and is less specific for residues at P1', with a decreasing preference for lysine, isoleucine, histidine and arginine (Dekker *et al.*, 2001). Furthermore, OmpT shows a high specificity for small hydrophobic residues (isoleucine, valine and alanine) at P2', and has a broad tolerance for residues at the P2 position (Dekker *et al.*,

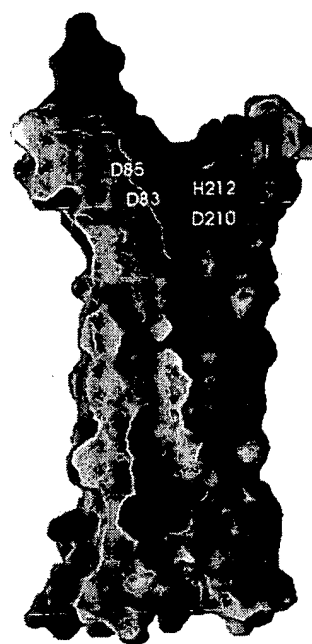


Fig. 4. Electrostatic surface potential of a cross-section of the  $\beta$ -barrel (orientation identical to Figure 1B). Negatively charged areas are shown in red, positively charged areas in blue. The positions of the proposed catalytic residues are labelled. This figure was produced by the program GRASP (Nicholls *et al.*, 1991).

2001). Given the high specificities for P1 and P2', we expect well-defined S1 and S2' subsites. A deep negative pocket containing Glu27 and Asp208 probably forms the S1 subsite, which determines the specificity for lysine and arginine at P1 (Figure 5). Approximately 7 Å away from this pocket, the groove is shallow and hydrophobic, with Met81 and Ile170 located at the bottom of the groove. This region may well form the S2' subsite, explaining the high specificity for small hydrophobic residues at P2'. Interactions between the substrate and the putative S1 and S2' subsites would position the scissile peptide bond between the Asp83–Asp85 and His212–Asp210 couples, in agreement with our proposed catalytic mechanism.

The current model implies a novel proteolytic mechanism, consistent with the observation that commonly used protease inhibitors do not or weakly affect the activity of OmpT. Solving the X-ray structure of OmpT in complex with a substrate analogue is needed to confirm our active site model and to provide more details on the catalytic mechanism. Since the active site is fully conserved among the omptins, the structure of OmpT may serve as a template to develop new types of antimicrobial compounds against omptin-mediated pathogenicity of these Gram-negative bacteria.

## Materials and methods

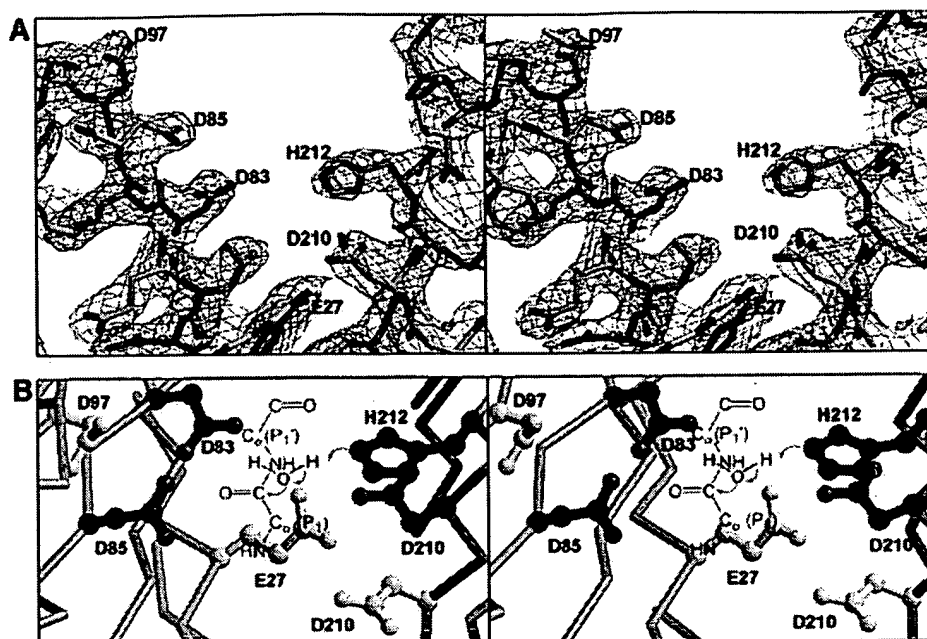
### Protein expression and purification

OmpT, containing the mutations S99A, G216K and K217G in order to abolish autoproteolysis (Kramer *et al.*, 2000b), was overexpressed without its signal sequence (of 20 residues) in *E.coli* and refolded as described previously (Kramer *et al.*, 2000b). For expression of Se-Met

				L1			
OmpT	STETLSFTPD	NINADISLGT	ESGMRKERV	LAEEGGKRVY	QLDAKFNHAA	IKKGAINRDL	60
OmpP	SDFFGEE	KISTEINLGT	LSGSTRKRV	EPEEGGKRVY	QLDKVSNHAA	ILKGAVNNEL	57
SopA	TTNYPLIFPD	NISTDISLGS	LSGSTRKRV	HPKEGGKRVY	QLDKVSNHAT	IVRGDILDKL	60
PgtE	ES ALFIFDVSPD	SVTISLSGV	LSGSTRKRV	TDOT-GRRLS	QLDKVSNHVA	TLGGDLSHP	61
Pla	AS 3QLFIHISPD	STTVAASTGM	ESGSTRKRV	DAET-GRRLS	QLDKVSNHVA	ILKGDISNDP	61
				L2			
OmpT	MPDISIGAAE	WTLGSRGGN	MYDGNMDSS	NPFGTWDESR	HPDTGLNVA	EFTDLNIGKH	120
OmpP	NFWLSVGAAG	WTLGSRGGN	MYDGNMDSS	TPQGTWDESR	HPDTGLNVA	EFTDLNIGKH	117
SopA	IFKVSFVSDS	WTLGSRGGN	MYDGNMDSS	TPQGTWDESR	HPDTGLNVA	EFTDLNIGKH	117
PgtE	YSFMTLDARD	WTLGSRGGN	MYDGNMDSS	QPG-WEDRS	HPDTGLNVA	EYDLNIGKH	120
Pla	YSFLTINARG	WTLGSRGGN	MYDGNMDSS	QSE-WEDRS	HPDTGLNVA	EYDLNIGKH	120
				L3			
OmpT	INEFNRLGL	MAGYQESRY	FTARGGSYV	SEEGGFRDPI	GSPNGERA	GKQCFEATY	180
OmpP	LKESDYRLA	MAGYQESRY	FTARGGSYV	SEEGGFRDPI	GALPKIKV	GKQCFEATY	177
SopA	ENLNDYRLG	MAGYQESRY	FTARGGSYV	SEEGGFRDPI	GAHPSEKT	GKQCFEATY	180
PgtE	LOGDNKAGV	TAGYQESRY	FTARGGSYV	-DN-G-A-YI	GAHPGVGRT	GKQCFEATY	178
Pla	LOGDNKAGV	TAGYQESRY	FTARGGSYV	-NN-G-A-YI	GAHPGVGRT	GKQCFEATY	178
				L4			
OmpT	IGLTGSRYE	DFELGTFKY	SWVAESND	IRYDQKRIT	YRSKVKDNY	YSVAVNAGY	240
OmpP	VGLTGNYRD	NFEFGAFKY	SWVAESND	IRYV--RQIT	FRSKVINQY	YSVAVNAGY	235
SopA	IGLTANYRHE	NFEFGAFKY	SWVAESND	IRYV--TETI	FKDSIKNSY	CSVAANIGY	238
PgtE	IGLACGYRN	DFELNALFY	SWVAESND	IRYV--RQIT	FRSKVINQY	YSVAVNAGY	238
Pla	IGLACGYRN	DFELNALFY	SWVAESND	IRYV--RQIT	FRSKVINQY	YSVAVNAGY	238
				L5			
OmpT	VIPNAKYVE	CAKRVYTHK	GTSLYDHNI	-NTSDYSKNG	AGLNYNFI	TAGLYTE	291
OmpP	VIPNAKYVE	CAKRVYTHK	GTSLYDHNI	-NTSDYSKNG	AGLNYNFI	TAGLYTE	291
SopA	VIPNAKYVE	CAKRVYTHK	GTSLYDHNI	-NTSDYSKNG	AGLNYNFI	TAGLYTE	291
PgtE	ITSNAKYVE	FAYSKYVE	GTSLYDHNI	GDYTFGGDA	AGLNYNFI	TAGLYTE	291
Pla	VIPNAKYVE	FAYSKYVE	GTSLYDHNI	GDYTFGGDA	AGLNYNFI	TAGLYTE	291

OmpT, *E. coli* strain B834(DE3) (Met-auxotroph, Novagen) containing the plasmid that encodes OmpT (S99A, G216K, K217G) was grown in new minimal medium (Budisa *et al.*, 1995) supplemented with 0.3 mM L-Se-Met (Acros). Refolded native OmpT and Se-Met OmpT (~200 mg) were loaded onto a 100 ml Fast Flow S-Sepharose column (Amersham Pharmacia) equilibrated with buffer A [10 mM Zwittergent 3–12 (Fluka, Switzerland), 20 mM sodium acetate pH 4.0]. The column was washed with buffer A and proteins were eluted with a linear gradient of NaCl to 1 M in buffer A. The native protein and the Se-Met derivative were concentrated to 25 mg/ml in buffer B (1% OG, 5 mM sodium acetate

Crystallization conditions for native OmpT were screened using the hanging drop vapour diffusion method at 4 and 20°C. Droplets containing equal amounts (1 µl) of protein and mother liquor were equilibrated against 0.5 ml of reservoir solution. Sparse-matrix screens for initial trials were performed using Hampton research screen kits (Hampton Research,



**Fig. 7.** Stereo view of the proposed catalytic site. (A) The final  $2F_o - F_c$  electron density map, at 2.6 Å resolution and contoured at  $1\sigma$  (orientation identical to Figure 1B). Residues are shown as sticks. (B) The catalytic site viewed from the top down the  $\beta$ -barrel axis. Active site residues are depicted by ball-and-sticks. Proposed catalytic residues are coloured dark grey and the other residues that are probably involved in substrate binding are coloured light grey. The proposed proteolytic mechanism, in which the His212–Asp210 couple abstract a proton from a water molecule which then attacks the main chain carbon, is represented schematically.

CA). After 6 months, one small crystal with dimensions of  $0.08 \times 0.05 \times 0.05$  mm appeared at 4°C in 30% (v/v) MPD (Fluka), 0.2 M ammonium acetate, 0.1 M sodium citrate pH 5.6. A narrower screening around this crystallization condition was performed: 28% (v/v) MPD, 0.5 M NaCl and 0.1 M sodium citrate pH 5.5 yielded one small crystal per drop within a month. Se-Met OmpT was produced in order to obtain phases for structure determination. Se-Met OmpT appeared to crystallize faster and yielded larger crystals than the native protein. After 2 weeks, crystals occurred in 1% OG, 30% (v/v) MPD and 0.3 M sodium citrate pH 5.5. These crystals grew to maximum crystal dimensions of  $-0.3 \times 0.2 \times 0.2$  mm in 3 months.

#### X-ray diffraction analysis

Crystals were harvested from the droplets with cryo-loops and directly frozen into liquid nitrogen. X-ray data were collected at 100 K on a CCD detector at the ID-14 EH4 beamline at the European Synchrotron Radiation Facility (ESRF) in Grenoble. Native data were collected to 2.6 Å, using an oscillation range of  $0.2^\circ$ . The crystal belongs to the trigonal space group  $P3_21$ , with unit cell parameters  $a = b = 98.4$  Å,  $c = 165.7$  Å,  $\alpha = \beta = 90^\circ$  and  $\gamma = 120^\circ$ . Crystals have a solvent plus detergent content of 63% (v/v) and two OmpT molecules in the asymmetric unit. A data set of a Se-Met OmpT crystal was collected at  $\lambda_{\text{peak}} = 0.9790$  Å to a resolution of 3.1 Å. Data were indexed using DENZO merged with SCALEPACK (Otwinowski and Minor, 1997) and processed further using truncate from the CCP4 suite (CCP4, 1994). A summary of the data collection and final processing statistics is provided in Table I.

#### Structure determination and refinement

Using the peak data set of Se-Met OmpT, all 10 selenium sites were found by direct methods with the DREAR/SnB package (Weeks and Miller, 1999). Phases subsequently were calculated using MLPHARE (Otwinowski, 1991) to 3.7 Å resolution. This yielded an  $R_{\text{cullis}}$  (ano) of 0.78 [ $R_{\text{cullis}}$  (ano) =  $\Sigma(|\Delta\text{PH}_{\text{obs}} - \Delta\text{PH}_{\text{calc}}|) / \Sigma|\Delta\text{PH}_{\text{obs}}|$  with  $\Delta\text{PH} = F_{\text{PH}}(+)-F_{\text{PH}}(-)$ ]. With density modification in CNS (Brünger *et al.*, 1998), phases were extended to 3.1 Å resolution. The resulting electron density map allowed the placement of 80% of the residues using the program O (Jones *et al.*, 1991). The native data set was used for refinement from 20 to 2.6 Å resolution. After a rigid body refinement,

iterative model refinement was performed by model building in O, followed by simulated annealing and restrained individual  $B$ -factor refinement in CNS. The final model consists of all residues of OmpT in molecule A, residues 11–297 in molecule B, four OG molecules and 29 water molecules. For the side chains of residues Glu3, Thr4, Glu154, Glu167, Arg168, Lys216 and Tyr266 of molecule A and Glu33, Glu154, Lys216 and Tyr266 of molecule B, no electron density was observed. Therefore, these side chains were omitted from the model. The two monomers in the asymmetric unit pack in a perpendicular orientation to each other and interact through their hydrophobic transmembrane regions. Intermolecular protein–protein interactions in the crystal are predominantly of a hydrophobic nature, with only one unique hydrogen bond (involving Arg100 in molecule A and the main chain oxygen of Ile160 in molecule B) and a salt bridge (involving Asp10 in molecule A and Lys277 in molecule B). In the Ramachandran plot, 85.4% of the residues are in most favoured regions, with 13.2% in additionally allowed regions, 1% in the generously allowed regions and one residue (Glu190) in a disallowed region. Ne of Arg188 is hydrogen bonded to the main chain oxygen of Glu190, generating a type II'  $\beta$ -turn, explaining the perturbed  $\phi, \psi$ -combination of Glu190. The overall temperature factor of the total structure is 53.2 Å<sup>2</sup>, with the highest  $B$ -factors in the extracellular loops (Figure 3).

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## **EXHIBIT 5**

# Identification of essential acidic residues of outer membrane protease OmpT supports a novel active site

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**Abstract** *Escherichia coli* outer membrane protease OmpT has previously been classified as a serine protease with Ser<sup>99</sup> and His<sup>212</sup> as active site residues. The recently solved X-ray structure of the enzyme was inconsistent with this classification, and the involvement of a nucleophilic water molecule was proposed. Here, we substituted all conserved aspartate and glutamate residues by alanines and measured the residual enzymatic activities of the variants. Our results support the involvement of a nucleophilic water molecule that is activated by the Asp<sup>210</sup>/His<sup>212</sup> catalytic dyad. Activity is also strongly dependent on Asp<sup>83</sup> and Asp<sup>85</sup>. Both may function in binding of the water molecule and/or oxyanion stabilization. The proposed mechanism implies a novel proteolytic catalytic site. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Active site; Asp/His dyad; Protease; OmpT; Outer membrane protein

## 1. Introduction

*Escherichia coli* OmpT (EC 3.4.21.87) is a 33.5 kDa outer membrane protease that cleaves peptides and proteins preferentially between two consecutive basic amino acids [1–5]. The enzyme has been suggested to be involved in urinary tract disease [6], in DNA excision repair [7] and in the breakdown of antimicrobial peptides [8], but its actual biological function remains to be elucidated. OmpT is a member of the omptin family that includes the proteases PgtE of *Salmonella typhimurium* (mature part 49% identical to OmpT) [9], Pla of *Yersinia pestis* (50% identical) [10], OmpP of *E. coli* (72% identical) [11] and SopA of *Shigella flexneri* (60% identical) [12]. As OmpT, Pla and OmpP lack cysteine residues, the omptins do not belong to the class of cysteine proteases. The omptin family appears to constitute a novel class of proteases, since

the amino acids conserved within the omptins are not part of known active site consensus sequences found in serine proteases, aspartic proteases or metalloproteases [13]. This is confirmed by the observation that OmpT activity is not or only slightly inhibited by commonly used class-specific protease inhibitors [1,14,15]. Significant inhibition of OmpT activity has been observed only at high concentrations of the serine protease inhibitors diisopropylfluorophosphate (DFP) or phenylmethylsulfonyl fluoride (PMSF) [1]. Members of the omptin family have been classified as serine proteases mainly based on these observations [16], even though several experiments have been reported where DFP and PMSF did not affect the proteolytic activity of OmpT [14,15,17].

In serine proteases, the scissile peptide bond is attacked by a nucleophilic hydroxyl of the catalytic serine that is usually activated by a histidine residue [13]. To identify the putative active site serine and histidine of OmpT, we previously substituted all conserved serines and histidines by site-directed mutagenesis and concluded that Ser<sup>99</sup> and His<sup>212</sup> are essential active site residues [18]. The activities of H212A, H212N and H212Q OmpT were reduced by four orders of magnitude. However, the mutation S99A in OmpT reduced activity only 500-fold, where at least a 10 000-fold reduced activity was expected (e.g. [19]). It was therefore emphasized that a role for Ser<sup>99</sup> other than performing the nucleophilic attack should not be excluded. To further examine the active site mechanism, we wished to study the possible involvement of residues other than serine and histidine. In the classical serine protease triad, a negatively charged aspartate residue is thought to stabilize the transient positive charge on the histidine during catalysis [13]. Recently, it was shown that a glutamate is able to perform this function as well [20]. Conserved acidic residues are thus obvious candidates for being an active site residue of OmpT. Furthermore, at least one aspartate or glutamate residue is expected to be involved in substrate binding, since OmpT specifically cleaves peptides between two positively charged amino acids [5]. OmpT contains 36 acidic residues, of which six aspartates (at positions 43, 83, 85, 97, 208 and 210) and five glutamates (at positions 27, 111, 136, 193 and 250) are fully conserved within the omptin family (Fig. 1). Using the same approach as described for the identification of Ser<sup>99</sup> and His<sup>212</sup> [18], these 11 residues were replaced by alanines and the residual activities of the resulting variants were measured. In parallel with these mutagenesis studies, we solved the X-ray structure of OmpT, which revealed a puta-

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**Abbreviations:** Abz, *o*-aminobenzoyl; Dap(dnp), *N*-β-dinitrophenyl-L-diaminopropionic acid; DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulfonyl fluoride; Triton X-100, polyethyleneglycol tert-octylphenyl ether; Tween 20, polyoxyethylene sorbitanmonolaurate

tive active site cleft at the top of the extracellular region [21]. Ser<sup>99</sup> and His<sup>212</sup> are both located in this cleft, but they are separated by 9 Å. The concerted action of Ser<sup>99</sup> and His<sup>212</sup> was seriously questioned and an alternative mechanism was proposed, involving an Asp<sup>210</sup>/His<sup>212</sup> catalytic dyad with a water molecule acting as nucleophile [21].

Here, we present the mutagenesis data of the conserved aspartate and glutamate residues and discuss their importance for the proteolytic activity of OmpT. The results support the involvement of an Asp<sup>210</sup>/His<sup>212</sup> catalytic dyad, suggesting that OmpT would be the first protease exhibiting this type of catalytic mechanism.

## 2. Materials and methods

### 2.1. Materials

DNA restriction enzymes were purchased from New England Biolabs. Oligonucleotides were bought from Amersham Pharmacia Biotech. Polyethyleneglycol tert-octylphenyl ether (Triton X-100) was obtained from Serva and polyoxyethylene sorbitanmonolaurate (Tween 20) was obtained from Bio-Rad. The fluorogenic peptide substrate Abz-Ala-Arg-Arg-Ala-Dap(dnp)-Gly (Abz = *o*-aminobenzoyl; Dap(dnp) = *N*-β-dinitrophenyl-L-diaminopropionic acid) was a kind gift of Mr. Ruud C. Cox (Utrecht University). Anti-OmpT monoclonal antibodies were obtained as described before [18]. Goat anti-mouse IgG alkaline phosphatase conjugate was purchased from Promega. All other chemicals were of analytical grade.

### 2.2. Bacterial strains and plasmids

*E. coli* K-12 strain DH5α [22] was used in the cloning procedures. *E. coli* strain BL21(DE3) [23] lacks the *ompT* gene and was used for expression. Plasmids pND9 and pND10 [4] are derivatives of pUC19 [24], containing the *ompT* gene, including its own promoter sequence oriented clockwise and counter-clockwise with the *lac* promoter, respectively.

All mutations were introduced using PCR according to the manufacturer's protocol provided with the QuikChange site-directed mutagenesis kit (Stratagene). The *ompT* gene contains only a few unique restriction sites. Therefore, four additional unique restriction sites were introduced to facilitate subcloning of introduced mutations. The sites *Xho*I (at Ser<sup>99</sup> of mature OmpT), *Sac*II (at Arg<sup>144</sup>), *Age*I (at Arg<sup>222</sup>) and *Eag*I (at Ala<sup>291</sup>) were introduced as silent mutations in plasmids pND10 and pND9, resulting in plasmids pRAK22 and pRAK23, respectively. Mutations resulting in the substitution of Asp or Glu by Ala were introduced individually into pRAK22. To facilitate the screening for introduced mutations, silent mutations resulting in the introduction or deletion of restriction sites were made concomitantly with the amino acid mutations. After verification of the correctly introduced mutations by DNA sequencing, all mutations were subcloned into pRAK23 using the newly introduced restriction sites. The sequences of the relevant parts of the constructed plasmids were checked by restriction enzyme analysis and DNA sequencing.

### 2.3. Expression, isolation and analysis of OmpT variants

After transformation by the various plasmids, BL21(DE3) cells were grown overnight in 10 ml LB medium [25] with 50 µg/ml ampicillin at 37°C. Membrane fractions containing OmpT were isolated from the bacteria, as described before [18], and solubilized in 200 µl buffer A (0.1% Triton X-100, 50 mM Tris, pH 7.5). The total amount of protein in the isolated membrane fractions was determined using the Bio-Rad protein assay with bovine serum albumin as a reference. Samples of membrane fractions corresponding to equal amounts of total protein were analyzed by Western blotting using anti-OmpT monoclonal antibodies, as described before [18].

### 2.4. Enzymatic activity assays

Samples of membrane fractions were diluted in buffer A to appropriate concentrations (varying from 0.5 µg to 0.2 mg of total protein per ml) prior to OmpT activity measurements. The internally quenched fluorogenic substrate Abz-Ala-Arg-Arg-Ala-Dap(dnp)-Gly was used in a fluorimetric activity assay, as described previously [4]. Assay conditions were 5 µM substrate, 1 mM Tween 20, 5 mM

EDTA, 10 mM Tris, pH 8.3. Activity was measured in a fluorimeter using excitation and emission wavelengths of 325 and 430 nm, respectively. After recording the initial linear increase in fluorescence, 20 µg of trypsin was added to determine the fluorescence of completely hydrolyzed substrate, which enabled quantification of the inner filter effect and allowed for the calculation of the activity in enzymatic units ( $U = \mu\text{mol}$  substrate converted per min).

## 3. Results

### 3.1. Expression of OmpT

Single mutant proteins of OmpT were constructed with the conserved acidic residues replaced by alanines. BL21(DE3) cells expressing either these alanine variants or wild-type OmpT were grown in triplicate and membrane fractions were isolated. Cells lacking a plasmid with the gene for OmpT were used as a control. The fact that isolated membrane fractions were used instead of purified protein had two important consequences. An important advantage of using outer membrane fractions was that all samples contained endogenous lipopolysaccharide, which has been shown to be required for OmpT activity [4]. Unfortunately the concentration of OmpT in the samples could not be accurately determined. Therefore, concentrations of total protein were determined for each sample and used as a measure for the amount of OmpT. We previously validated this method for serine and histidine variants of OmpT by showing that the expression levels of these variants were comparable to that of wild-type OmpT [18]. To check whether this was also true for the aspartate and glutamate variants, SDS-PAGE and Western blot analysis were performed with all samples containing equal amounts of total protein. As expected, the expression levels of the variants and wild-type OmpT were comparable within a factor of three (data not shown). These differences are acceptable in our studies, since activities reduced by at least two orders of magnitude are expected when essential residues are replaced. SDS-PAGE and Western blotting were also used to check the correct folding of the variants. At room temper-

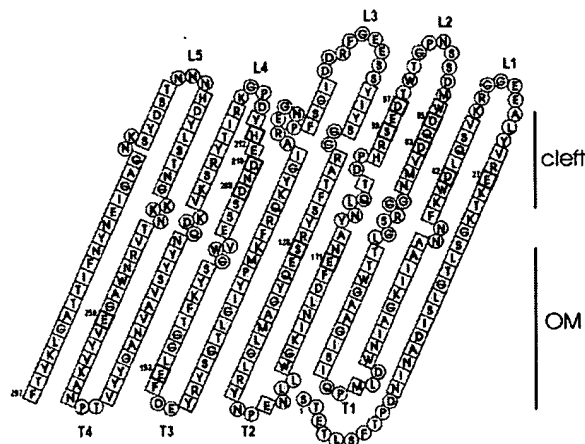


Fig. 1. Topology of OmpT (adapted from [21]). Numbers refer to amino acid positions in mature OmpT. Residues in  $\beta$ -strands are shown in squares; other residues are in circles.  $\beta$ -Strand residues pointing to the protein exterior are in shaded squares. Conserved acidic residues, as well as Ser<sup>99</sup> and His<sup>212</sup>, are in squares with dark edges. The approximate positions of the outer membrane (OM) and the putative active site cleft of OmpT are indicated. The N-terminus and the short turns (T1-T4) are localized in the periplasm, and the large loops (L1-L5) face the extracellular side of the membrane.

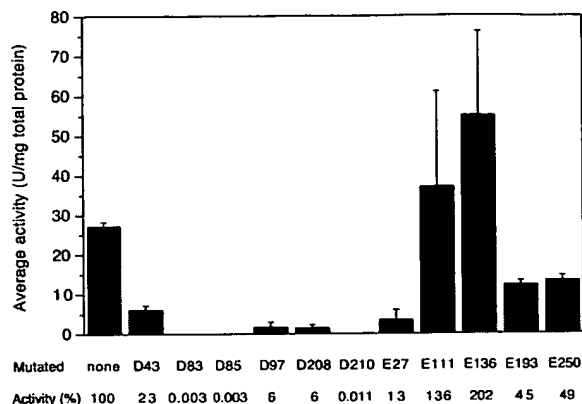


Fig. 2. Enzymatic activities of OmpT and its variants as measured in a fluorimetric assay. Average absolute activities of three independent measurements (black bars) and the corresponding relative activities compared to wild-type OmpT (percentages) were determined for each variant. See the text for experimental details.

ature, the presence of SDS is not sufficient to unfold the  $\beta$ -barrel conformation of OmpT. As a consequence, native OmpT has a more compact shape than the heat-denatured protein and runs faster on SDS-PAGE gel [4,18]. This difference in migration rate between folded and unfolded protein is known as heat-modifiability, which is generally observed for outer membrane proteins [26]. The Western blots showed that all of the 11 active site variants displayed this effect (data not shown), indicating their proper folding and assembly in the outer membrane.

### 3.2. Activity measurements of OmpT variants

The enzymatic activity of OmpT in the isolated membrane fractions was determined using a fluorimetric assay. For all

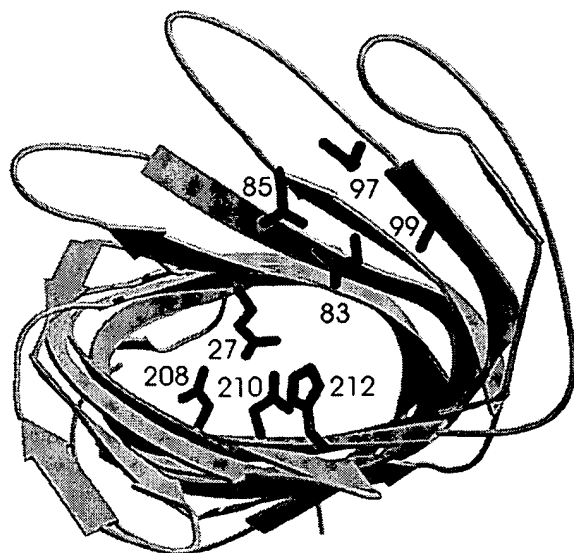


Fig. 3. Active site cleft of OmpT (adapted from [21]). OmpT (ribbon presentation) is viewed parallel to the barrel axis from the extracellular side of the outer membrane. The conserved acidic residues Glu<sup>27</sup>, Asp<sup>83</sup>, Asp<sup>85</sup>, Asp<sup>97</sup>, Asp<sup>208</sup> and Asp<sup>210</sup>, as well as Ser<sup>99</sup> (Ala<sup>99</sup> in the crystallized protein) and His<sup>212</sup>, are represented as stick model.

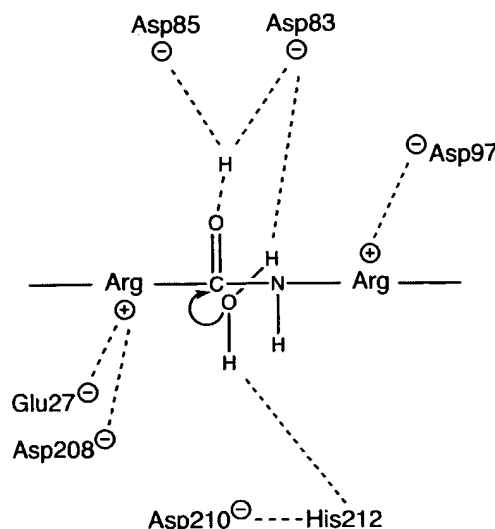


Fig. 4. Schematic two-dimensional model of a peptide in the active site of OmpT. Hydrogen bonds and ionic interactions are indicated by dashed lines. The curved arrow represents the nucleophilic attack on the carbonyl carbon of the scissile peptide bond.

variants, membrane fractions of three independent cell cultures were measured. The average activities are shown in Fig. 2. A large spread in the activities was observed for several variants, which was at least partially due to variations in expression levels as mentioned above. Variants E111A, E136A, E193A and E250A displayed enzymatic activities that were comparable to wild-type within a factor of two. In contrast, variant E27A, as well as all aspartate variants, showed significantly reduced activities compared to wild-type OmpT. Specific activities of the variants E27A, D43A, D97A and D208A were reduced 4–17-fold. The strongest decrease was observed for variants D83A, D85A and D210A, exhibiting at least 10 000-fold lower activity than wild-type OmpT.

### 4. Discussion

The experimental basis for the current classification of OmpT as a serine protease is weak, as pointed out in Section 1. To investigate the nature of the active site, we studied the involvement of acidic residues in substrate binding and/or hydrolysis by constructing single mutant proteins of OmpT. Substitution of the conserved residues Asp<sup>43</sup>, Glu<sup>111</sup>, Glu<sup>136</sup>, Glu<sup>193</sup> or Glu<sup>250</sup> by alanines did not lead to severe reductions in proteolytic activity (Fig. 2). Not surprisingly, the side chain of none of these residues is located in the putative active site cleft (Fig. 1) [21]. Deleting either of the remaining six conserved acidic residues, which are all located inside the highly negatively charged cleft (Fig. 3) [21], resulted in at least seven-fold lower activity. OmpT preferentially cleaves peptides between two basic amino acids [5], therefore the anionic nature of the cleft is probably a major determinant of its substrate specificity. More specifically, we proposed earlier, based on the crystal structure of OmpT [21], that Glu<sup>27</sup> and Asp<sup>208</sup> may define the high specificity of OmpT for Arg or Lys at position P1 in the substrate (nomenclature as in reference [27]). The mutagenesis data reported here are in good agreement with this proposal. For the P1' position, the specificity was less exclusive, but a positively charged amino acid was

preferred there as well [5], likely due to interaction with an anionic residue of OmpT. Assuming that the substrate has an extended conformation and that the P1 side chain points towards Glu<sup>27</sup> and Asp<sup>208</sup>, the P1' chain would be located close to Asp<sup>97</sup>. D97A OmpT displayed only 6% residual activity, therefore we propose that Asp<sup>97</sup> is responsible for the observed P1' specificity. A schematic model of a peptide in the active site is shown in Fig. 4.

Substituting the aspartate at position 83, 85 or 210 reduced activity at least 10000-fold, suggesting that these residues may participate directly in enzymatic catalysis. The absolute requirement for more than one aspartate is reminiscent of the aspartic proteases, in which two aspartates activate a nucleophilic water molecule [13]. The relative orientation of Asp<sup>83</sup> and Asp<sup>85</sup> is in fact similar to that in aspartic proteases [21]. However, OmpT does not contain the D(T/S)G consensus sequence of aspartic proteases and it is not active at acidic pH [4], while the overall fold is very different from aspartic proteases [13]. A possible role for the Asp<sup>83</sup>/Asp<sup>85</sup> couple is discussed below. The X-ray structure of OmpT showed that the relative orientation of Asp<sup>210</sup> and His<sup>212</sup> is similar to that in the classical Ser/His/Asp triad and that there is no density at the position where a serine would be expected (Fig. 3) [21]. Based on this information, we recently postulated that OmpT has an Asp<sup>210</sup>/His<sup>212</sup> catalytic dyad with a water molecule acting as nucleophile [21]. The observation that Asp<sup>210</sup> is essential for activity supports this hypothesis. The existence of an Asp/His catalytic dyad has been proposed for several enzymes catalyzing hydrolytic reactions, including phospholipase A2 [28], phosphoesterases [29], endonucleases [30], endocellulases [31] and haloalkane dehalogenase [32]. Interestingly, it has never been observed in proteases, implying that OmpT would be the first example of a protease using this type of catalytic mechanism. As amide bonds are relatively stable, other factors are expected to facilitate peptide cleavage by activated water. Presumably the Asp<sup>83</sup>/Asp<sup>85</sup> couple plays a role in the catalytic mechanism, for example by coordinating the nucleophilic water molecule as proposed before [21]. In this way, Asp<sup>83</sup> and Asp<sup>85</sup> would be indirectly involved in activation of the water molecule, a mechanism that is different from the direct activation of water by aspartates in aspartic proteases. Alternatively or in addition, a proton shared by the carboxyl moieties of Asp<sup>83</sup> and Asp<sup>85</sup> might stabilize the oxyanion intermediate during the reaction. In agreement with these hypotheses, the mutagenesis experiments show that both amino acids are required for activity. Further insight in the role of the essential residues Asp<sup>83</sup>, Asp<sup>85</sup> and Asp<sup>210</sup> might be obtained by determining the kinetic parameters  $k_{cat}$  and  $K_M$  of the corresponding OmpT variants. Unfortunately though, the residual activities of D83A, D85A and D210A OmpT were very close to the detection limit of our assay, making it impossible to obtain reliable Michaelis–Menten curves for these variants. The preferred route to detailed elucidation of the active site mechanism would be to determine the X-ray structure of OmpT in the presence of a peptide substrate. Attempts to crystallize OmpT in complex with a substrate analogue are currently under way.

Prior to submission of this paper, we became aware of a very recent publication on ompT of *Y. pestis* by Kukkonen et al. [33], who observed proteolytic importance for Pla residues corresponding to Ser<sup>99</sup>, His<sup>101</sup>, His<sup>212</sup>, Asp<sup>83</sup>, Asp<sup>85</sup> and Asp<sup>210</sup> in OmpT. They could not settle the exact roles of

these residues, but hypothesized that Pla may contain a Ser<sup>99</sup>/His<sup>212</sup>/Asp<sup>210</sup> triad (OmpT numbering), whereas Asp<sup>83</sup>, Asp<sup>85</sup> and His<sup>101</sup> were proposed to be involved in substrate interaction [33]. Now that we have solved the crystal structure of OmpT and generated mutagenesis data on all conserved Ser, His, Asp and Glu residues in OmpT, we have a sound basis for our novel active site model, which does not involve a nucleophilic serine. The fact that the proposed catalytic residues Asp<sup>83</sup>, Asp<sup>85</sup>, Asp<sup>210</sup> and His<sup>212</sup> of OmpT have proteolytically important counterparts in Pla, indicates that all ompTins likely use the same catalytic mechanism.

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